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Sensitization to Trail-Induced Apoptosis in K-RAS 12 Mutant Pancreatic Cancer Cells by BITC

Christina Ann Wicker

Wright State University

SENSITIZATION TO TRAIL-INDUCED APOPTOSIS IN K-RAS 12 MUTANT PANCREATIC CANCER CELLS BY BITC

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

CHRISTINA ANN WICKER
B.S. , Wright State University, 2006

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Christina Ann Wicker ENTITLED SENSITIZATION TO TRAIL-INDUCED APOPTOSIS IN K-RAS 12 MUTANT PANCREATIC CANCER CELLS BY BITC BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

_____

Thomas L. Brown, Ph.D.
Thesis Director

_____

Timothy Cope, Ph.D.
Department Chair

Committee on
Final Examination

_____

Thomas L. Brown , Ph.D.

_____

Nancy Bigley, Ph. D.

_____

Courtney Sulentic, Ph.D.

_____

Joseph F. Thomas, Jr., Ph.D.
Dean, School of Graduate Studies

ABSTRACT

Wicker, Christina Ann. M.S., Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2008. Sensitization to TRAIL Induced Apoptosis in K-Ras 12 Mutant Pancreatic Cancer Cells by BITC.

Pancreatic adenocarcinoma is an aggressive cancer with a greater than 95% mortality rate and short survival after diagnosis. Chemotherapeutic resistance hinders successful treatment. This resistance is associated with mutations within codon 12 of the K-Ras gene (K-Ras 12), which is present in over 90% of all pancreatic adenocarcinomas. Codon 12 mutations maintain Ras in a constitutively active state leading to continuous cellular proliferation.

Our study determined if TRAIL resistance in pancreatic adenocarcinomas with K-Ras 12 mutations could be overcome by first sensitizing the cells with Benzyl isothiocyanate (BITC). BITC is a component of cruciferous vegetable extracts and a cell cycle inhibitor. BxPC3, MiaPaCa2, and Panc-1 human pancreatic adenocarcinoma cell lines were examined for TRAIL resistance. Our studies show BITC-induced TRAIL sensitization by the activation of caspases 8, 9 and 3 as well as their respective substrates Bid, XIAP, and PARP. Cell Death ELISA confirmed TRAIL sensitization by BITC.

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I would of course like to especially thank Dr. Brown for the opportunity to work in his lab. He was very patient when I had a rough start and for whatever reason my experiments just stopped working because quite honestly if I was in his position I think I would have thrown my butt out of the lab. So I hope that one day, when I have people working under me that I will have this same patience as I think I managed to turn out O.K. He also has the very rare ability to get people to work and work hard, without being demeaning or abusive, which again I hope to hold to this. I have learned so much in such a short amount of time, not just technical skills, but how to think like a scientist. Skills that will aid me throughout my career and that combined with my drive will hopefully lead to me finding my place in science.

I would also like to thank Kashmira Kulkarni-Datar, as she was the one who had to put up with me when I was somewhat resistant to relearn what I thought I already knew. She taught me most of my technical skills and she is whom I look up to in hopes that one day I will be as technically gifted and as efficient and productive as she is. Even though I got the "whacking stick" a few times.

I spent more time with the members of my lab than my own family and in turn they became a second family to me. They supported me through issues in the lab and even some rather difficult issues I had outside the lab and for this I will be forever grateful. For without them, I am not sure I would have gotten through them.

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I would like to specially thank the Putnam lab for allowing me to borrow SDS-PAGE glass plates to finish my Western blots, after many of the plates in our lab came to an unfortunate end by numerous means. I would like to thank Dr. Lynn Hartzler for putting the idea in my head to go to the Experimental Biology in San Diego. They also allowed me to stay with them in San Diego for Experimental Biology 2008, which meant I could actually afford to go. It allowed me to travel on an airplane for the first time, cross the country, see the ocean for the first time and finally after 15 years of waiting to see and pet a real live dolphin. And the pilot on the way in took the time to do a turn-by-

turn tour of the Grand Canyon, which I think is the most awe inspiring thing I have seen in my entire life. Oh yeah, the conference was good too. No really it was, it was amazing to see a sea of posters and there were many career centered talks, which will continue to help me for many years as I set up my career as a scientist.

I also want my friends and family to know how much I appreciate their support during the past two years. Most of them have understood why I virtually abandoned them the past two years and why I was completely oblivious as to world news and other important facts such as who won American Idol this season and why I no longer had the time or money to go to Marcon, Midohiocon and so on. I would especially like to thank my grandmother for allowing me to mooch off of her during both undergraduate and graduate school, for I am not sure I could have afforded to go otherwise. I would likely still be working on my B.S instead of receiving my M.S. Even though my grandfather past some years ago, he was truly a second father to me and I try to work hard to this day so that he would be proud of what I have accomplished. I would like to thank my sisters, both of my mothers and father for being there when I just needed someone to talk to. I would especially like to thank my mother, who especially during the past few weeks, has taken extra effort to provide support and stress relief while I try to finish this paper and a laundry list of other tasks within a ridiculously short amount of time.

I would finally like to thank my Aunt Leona Fouts, who will never know how much she kept me going when nothing was working because she passed away from pancreatic cancer just shortly before I started in Dr. Brown's lab. Although I have never been much a believer in these type of things, it just seemed like more than a coincidence that the lab I wanted to work in since I was a junior, was just about to start working on

pancreatic cancer so shortly after she had passed. I continued to work hard despite fallbacks, so that I had the opportunity to continue working on this project. I was never delusional to think I could cure this disease but I hope like many other researchers will that I found a small piece to the puzzle that will eventually complete what is needed to greatly extend the lives of people stricken with pancreatic cancer.

I. INTRODUCTION

Pancreas

The pancreas is a small finger-like organ located behind the stomach. It a vital organ and has both endocrine and exocrine functions. Endocrine functions occur within the islets of Langerhans where alpha cells produce glucagon, beta cells produce insulin and delta cells produce somatostatin (1). These products are integral in maintaining blood glucose homeostasis. Glucagon facilitates the breakdown of glycogen that the liver stores to increase blood glucose levels and insulin is responsible for glucose uptake into cells. Somatostatin is an inhibitory hormone with a wide variety of functions including inhibition of hormones associated with gastric motility and can also inhibit insulin and glucagon release (2). Exocrine functions of the pancreas are in relation to food digestion. Bicarbonate is secreted by centroacinar cells into the small intestine to neutralize stomach acids (1). Within the pancreatic acini, digestive enzymes are produced. Inactive proteases such as trypsinogen and chymotrypsinogen are necessary for protein digestion. The pancreas also secretes pancreatic lipase for digestion of fats and pancreatic amylase for starch digestion (1). All of these functions are critical for normal digestion and blood glucose homeostasis and disruption of these functions such as by pancreatic cancer lead to severe consequences.

Pancreatic Cancer

Pancreatic cancer is the fourth leading cause of cancer related deaths in the United States (3,4). It is estimated that in 2008, there will be 37,680 new cases of pancreatic cancer (3,4). Incidence is nearly equal in both the female and male population (3). Risk factors for pancreatic cancer include age, obesity, diabetes, hereditary chronic pancreatitis, and exposure to carcinogens, such as those released during smoking (5,6). Pancreatic cancer includes exocrine, neuroendocrine and adenocarcinoma. Adenocarcinoma is the most common and has the least favorable prognosis (7,8).

In general, patients remain asymptomatic until wide spread metastasis has occurred. Symptoms that do appear later include fatigue, weight loss, jaundice, back and/or abdominal pain (3). There are three usual treatment options including surgery, radiation, and chemotherapy. Surgical options include pancreatic resection or complete pancreatectomy; however, these options are only available to 20% of patients (9). Pancreatic adenocarcinomas are largely resistant to both radiation and chemotherapy. Current chemotherapeutic options include the use of DNA replication inhibitors such as Gemcitabine, 5-Fluorouracil (5-FU) as well as platinum-based drugs including Cisplatin, which also inhibit DNA replication (10). Other therapies include inhibition of growth factors associated with tumor growth, such as epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) but these show limited effectiveness to date (10). The combination of late detection and chemotherapeutic resistance in pancreatic cancers is likely responsible for a greater than 95% mortality rate (3,4).

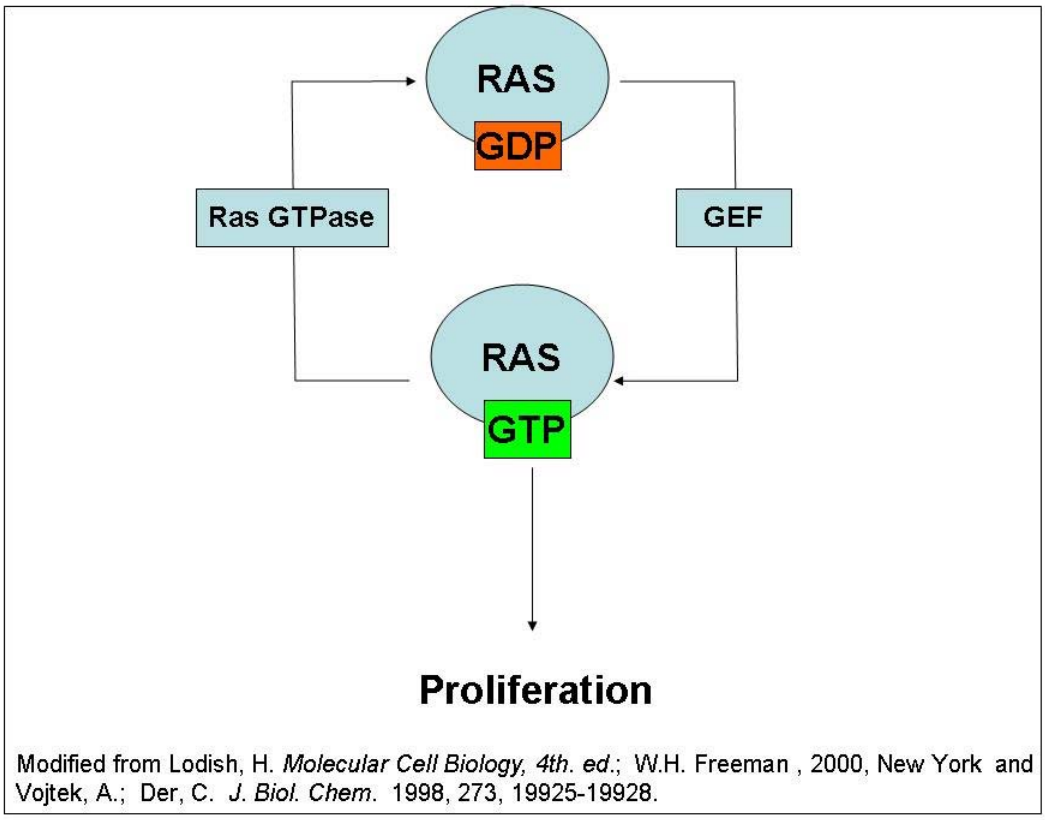
K-Ras 12

Over ninety percent of pancreatic adenocarcinomas harbor a mutation within codon 12 of the K-Ras gene (11,12). Although other mutations, such as p53, p16, and SMAD 4 have been reported in pancreatic adenocarcinomas, K-Ras mutations have been shown to be an initiating factor in the formation of pancreatic cancers (12). Ras regulates migration, cytoskeletal formation, apoptosis, and cellular proliferation amongst other functions, predominantly through Erk 1/2 signal transduction pathways (13-15). The Ras family includes three Ras isoforms Harvey (H-Ras), Kirsten (K-Ras) and Neuroblastoma (N-Ras) (16-18). Differences between these genes are largely in the C-terminal region (19,20). All Ras isoforms are involved in signaling through MAPK and AKT pathways, although some isoform-specific signaling has been reported (21).

Ras is a G protein that cycles between an inactive GDP-bound state and an active GTP-bound state (Fig. 1) (19). The Guanine-Nucleotide exchange factor (GEF) assists in the dissociation of GDP allowing GTP binding and thus activating Ras (22). GTPases such as the intrinsic GTPase within the Ras structure and Ras-GAP hydrolyse GTP to GDP and thus inactivates Ras (23,24). Mutations within codon 12 of K-Ras hinder this endogenous GTPase activity, thereby maintaining Ras in its GTP-bound active state (17). Constitutive activation of Ras leads to continuous cellular proliferation.

Figure 1. Ras cycling and signaling. Ras cycles between an inactive GDP bound state and an active GTP bound state. This cycling is facilitated by GEF and GTPases.

Figure 1. Ras cycling and signaling



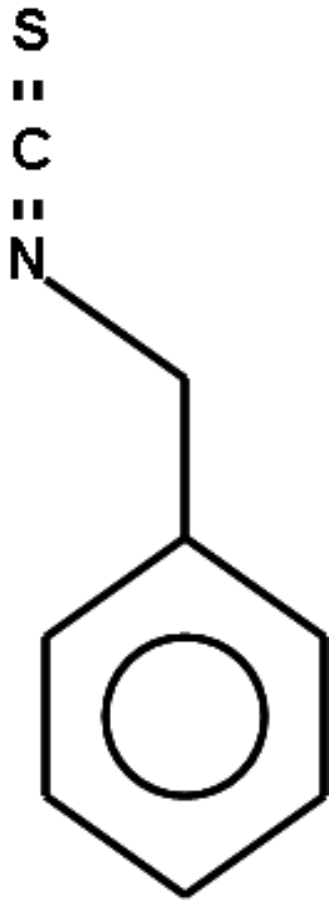
Modified from Lodish, H. *Molecular Cell Biology, 4th. ed.*; W.H. Freeman , 2000, New York and Vojtek, A.; Der, C. *J. Biol. Chem.* 1998, 273, 19925-19928.

BITC

Previous reports have shown that BITC can inhibit cell cycle progression and initiate pancreatic cancer cell death (25). BITC is a cruciferous vegetable extract and is a member of the isothiocyanate family. Isothiocyanates have been found to be protective against carcinogenesis by several mechanisms (25,26). These include modulation of phase I and II p450 enzymes and the cell cycle. Cytochrome P450, a phase I enzyme, has also been implicated in carcinogen activation. BITC has been shown to inhibit P450 and its role in carcinogenesis (27). Cytochrome P450 is involved in oxidative metabolism, which may lead to the formation of ROS (28, 29). ROS have been implicated in tumorogenesis through DNA damage, epigenetic changes, and supporting angiogenesis needed for tumor growth (30-32). BITC has been shown to induce Phase II enzymes including glutathione transferase, which can detoxify hazardous metabolites that may initiate carcinogenesis (27,29,33). BITC has an electrophilic structure and it is attracted to nucleophiles such as GSH, which are at high intercellular levels (Fig. 2) (34). Isothiocyanates have been implicated in mitochondrial membrane damage, which can initiate the intrinsic mitochondrial cell death pathway (35). The third method against carcinogenesis involves BITC increasing G2/M cell cycle arrest by decreasing Cdk1, CyclinB1, and Cdc25C protein levels (25,36-40).

Figure 2. BITC structure. This figure illustrates the structure of BITC. The –N=C=S is responsible for the compounds electrophilic properties, meaning the compound is attracted towards negatively charged compounds.

Figure 2. BITC structure



TRAIL

K-Ras 12 mutations have been associated with chemotherapeutic resistance to TRAIL-induced apoptosis. TRAIL (TNF related apoptosis-inducing ligand) is a novel and potential chemotherapeutic agent. TRAIL is both membrane bound and secreted. It is produced in nearly all antigen presenting cells (APC) (41-43). There is debate over TRAIL's normal physiological role in humans but some propose it as a guard against tumorogeneis (44).

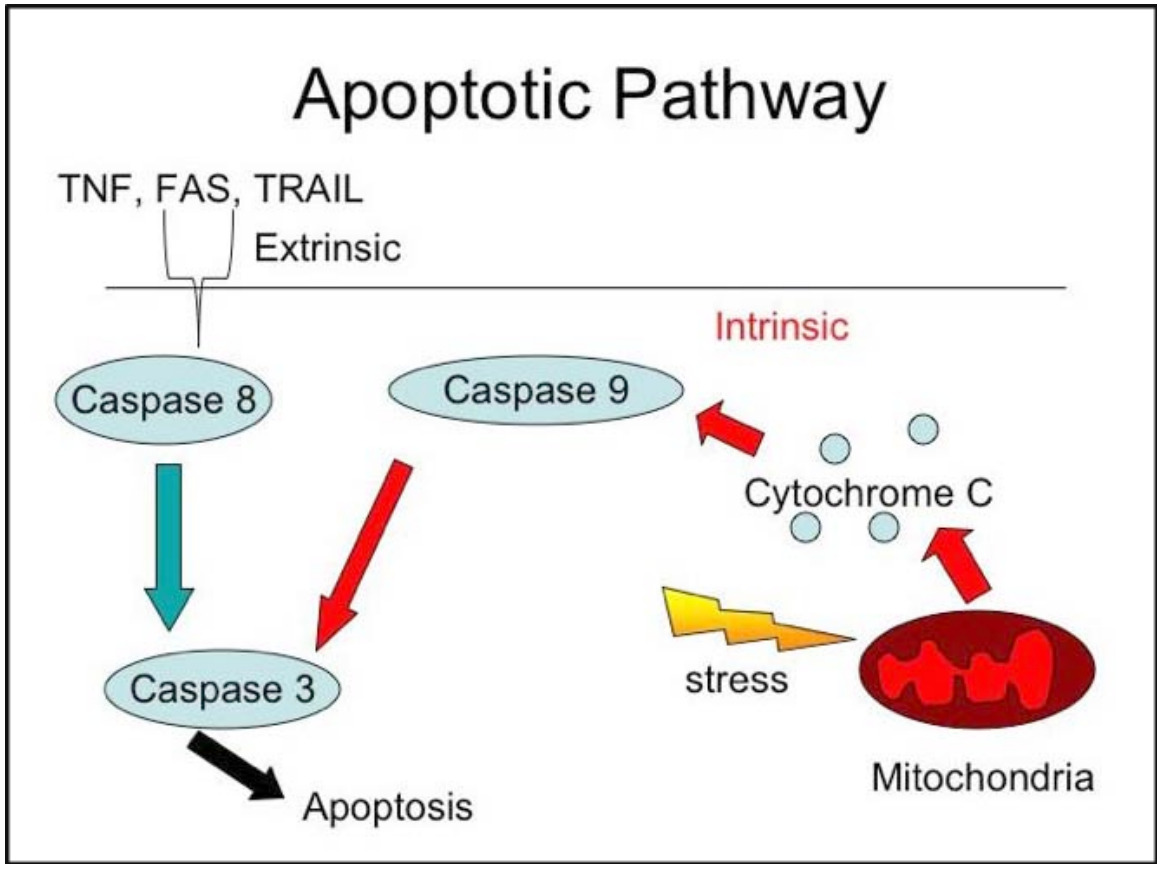
TRAIL is a member of the TNF cytokine family. Five TRAIL receptors including 2 decoy receptors DCR1 and DCR2, two death receptors DR4 and DR5 and an osteoprotogerin (OPG) receptor exist. The decoy receptors bind to TRAIL but lack the intercellular signaling needed to induce cell death. It has been proposed that these receptors act to bind up TRAIL and protect the tissues from death (45). Cell death can be induced when TRAIL binds to DR4 and DR5 receptors. OPG binds to TRAIL but does not induce cell death as it lacks the needed intracellular signaling. It is thought to also act as a decoy receptor in the adult. TRAIL death receptors are highly expressed on transformed cells but generally absent on most normal cells (46). The cytotoxic effects of TRAIL are pronounced on cancerous cells but leave most noncancerous tissues unaffected (47,48). TRAIL has been shown to induce apoptosis in sensitive cells (49). Previous studies have attempted to use TRAIL as a chemotherapeutic agent in human pancreatic cancer cells. However, overall cells exhibited similar resistance as other chemotherapeutics (49,50).

Apoptotic Pathway

Two major pathways, the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway regulate apoptosis (Fig. 3)(46). The extrinsic cell death pathway begins with external death receptors on the cell surface. When ligands such as TNF alpha, Fas or TRAIL have bound to their specific receptor, intercellular signaling occurs through recruitment of adaptor proteins (51,52). Recruitment of adaptor proteins induces caspase 8 cleavage and activation. Caspase 8 can cleave effector caspase 3 inducing apoptosis directly (53). Alternatively, caspase 8 can trigger the activation of the intrinsic cell death pathway by cleavage of Bid, a proapoptotic protein (54). Truncated Bid (t-Bid) is capable of interacting with other pro-apoptotic proteins leading to loss of mitochondrial membrane integrity, which has been shown previously to release cytochrome C (35). Cytochrome C release is associated with the activation of caspase 9 (55). Activated caspase 9 can lead to cleavage and activation of effector caspase 3 and induce apoptosis (53,56-60).

Figure 3. Extrinsic and intrinsic pathways of apoptotic death. This figure depicts signaling of the extrinsic and intrinsic apoptotic pathway and its key components.

Figure 3. Extrinsic and intrinsic pathways of apoptotic death



Rationale

In pancreatic cancer, K-Ras 12 mutations have been correlated to increased resistance to chemotherapeutic agents including TRAIL (61). These mutations cause Ras to remain in its active GTP-bound state causing continual cellular proliferation (17). BITC is capable of inducing cell cycle arrest in human pancreatic cancer cells (25,63). Pretreatment of cells with BITC to inhibit Ras activity may restore sensitivity to TRAIL.

Focus of Study

BxPC3, MiaPaCa2, and Panc-1 cell lines were selected because they represent the most common mutations found in human pancreatic adenocarcinomas (Table 1) (62,64,65). BxPC3 cells, isolated from human pancreatic adenocarcinoma, are tumorigenic but are wildtype for codon 12 K-Ras. Panc-1 cells, harbor a glycine to aspartate amino acid change within codon 12, and MiaPaCa2 cells, a glycine to cysteine amino acid change were also studied. We hypothesize that BITC will sensitize mutant K-Ras 12 human pancreatic adenocarcinoma cell lines to TRAIL-induced apoptosis. Our results indicate that BITC sensitizes chemotherapeutically resistant human pancreatic adenocarcinoma cells to TRAIL-induced apoptosis.

Table 1. K-Ras 12 Mutations in BxPC3, MiaPaCa2, and Panc-1 Human Pancreatic Cancer Cell Lines

Cell Line	K-Ras 12 Nucleotide Change	Amino Acid Change	References
BxPC3	GGT	None; Gly	(61,62)
MiaPaCa2	GGT→**T**GT	Gly→Cys	(61,62)
Panc-1	GGT→G**A**T	Gly→Asp	(61,62)

Significance

Pancreatic cancer is one of the top 5 deadliest cancers in the U.S. and is virtually incurable (3). Current treatments are mostly palliative in nature and patients are not expected to live much more than 6 months after diagnosis. K-Ras 12 mutations, present in over 90% of adenocarcinomas, are heavily correlated with chemotherapeutic resistance (11,12,66). This resistance is a major component of the greater than 95% mortality rate (3,4). Our study demonstrates one method of combating chemotherapeutic resistance associated with K-Ras 12 mutations in pancreatic adenocarcinoma.

II. MATERIALS AND METHODS

Materials

Panc-1 and BxPC3 cells were purchased from ATCC. Dr. B. Weissman of University of North Carolina, Chapel Hill School of Medicine kindly provided MiaPaCa2 cells. Goat anti-rabbit (W401B) and goat anti-mouse (W402B) HRP-conjugate secondary antibodies were purchased from Promega. The primary antibodies used were rabbit polyclonal anti-XIAP (Cell Signaling Cat # 2042), rabbit polyclonal PARP (Cell Signaling Cat # 9541S), mouse monoclonal caspase 8 (Cell Signaling Cat # 9746), active caspase 9 mouse monoclonal (Upstate Biotech Cat # 05-572), and Bid rabbit polyclonal (Cell Signaling 2002). Pan actin mouse monoclonal was kind gift from Dr. J. Lessard at Cincinnati Childrens Hospital. Active caspase 3 rabbit polyclonal antibody was a kind gift from Dr. K. Tomaselli at Idun Pharmaceuticals. For apoptotic death quantification, the Roche Cell Death ELISA Assay was used (Roche Cat # 1 774 425). Human recombinant TRAIL was purchased from R&D Systems (Cat # 375-TEC). BITC was purchased from Sigma-Aldrich (Cat # 252492). Anti-fas antibody was purchased from Upstate Biotech Cat # 05-201.

Cell Culture

BxPC3, MiaPaCa2, and Panc1 cells were maintained in RPMI 1640 with L-glutamine, supplemented with 5% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic. Cells were maintained at 5% CO_2 and 37° C. All cell lines were maintained at $1X10^5$ cells/ml.

Treatment Protocol

For collection of whole cell lysates used in Western blot analysis, cells were plated at $2X10^5$ cells/ml and allowed to sit for overnight. The next evening, media was changed to 5 ml RPMI 1640 with 5% FBS and 1 % antibiotic/antimycotic. Vehicle and TRAIL alone groups were treated with 5 μl 50 % DMSO/ddH_2O. BITC and BITC with TRAIL groups were treated with a final concentration of 5 μM BITC overnight. The following morning vehicle and BITC groups were treated with 5 μl 0.1% BSA. TRAIL and BITC combined with TRAIL groups were treated with a final concentration of 10 ng/ml TRAIL for 6 hours. Total treatment time with BITC was 24 hours. Cells were then collected for whole cell lysates.

As a positive control, Jurkat cultures were treated at $1X10^6$ cells/ml with 100 ng/ml anti-Fas antibody for 4 hours or 1 μg/ml Actinomycin D for 4 hours and whole cell lysates were collected for Western blot analysis.

Western Blots

Cell lysates were collected in CHAPS buffer (50 mM PIPES pH 6.5, 2 mM EDTA pH 8.0, 0.1 % CHAPS) with proteosome inhibitor, sonicated, and centrifuged at 12,000 rpm for 10 minutes at 4°C. Protein content was determined using the Bradford assay (67). Cell lysates were treated with Reducing Sample Buffer, and heat-treated for 5 minutes at 100°C. Proteins were resolved on an SDS polyacrylamide gel via electrophoresis (57-60). Separated proteins were subsequently transferred onto PVDF membrane in Western transfer buffer overnight at 0.1 A (68). Proteins were visualized using Ponceau S staining and the membrane was then blocked at room temperature for one hour in milk blocking buffer (1X PBS, 5% (w/v) non-fat dry milk, and 0.05 % Tween-20) for caspase 3 and 9 and Bid. Blots were blocked in TBS milk blocking buffer for XIAP, PARP, and caspase 8. Blots were rinsed in 1X PBS or 1XTBS containing 0.5% Tween-20. XIAP, PARP, caspase 8 and 9 were incubated with the primary antibody overnight at 4°C. Actin was incubated at (1:16,000) for 40 minutes at room temperature (RT) and active caspase 3 at (1:4,000) for 2 hours at RT. Primary antibodies include Bid (1:1,000), XIAP (1:1,000), cleaved PARP (1:1,000), active caspase 8 (1:1,000) and active caspase 9 (1:1,000). Blots were incubated with secondary antibody for 1.5 hours at room temperature. Secondary antibodies included goat anti-rabbit (1:50,000) and goat anti-mouse (1:10,000). For actin, goat anti-mouse was used at (1:20,000). Blots were then incubated following the manufacturer's recommendations with Pierce SuperSignal® West Pico Chemiluminescent Substrate and exposed to film or imaged on Fugi using LAS 3000 software.

Cell Death ELISA Assay

Roche Cell Death ELISA Assay kit was used after treatment of TRAIL to detect increase in oligosomal fragments that are indicative of apoptosis. This assay uses a sandwich ELISA method, whereby streptavadin-coated plates are pretreated with an anti-histone antibody. Treated lysate is applied the plates are then rinsed to remove any unbound DNA. Anti-DNA-peroxidase antibody is then applied to bind to the bound oligonucleosomal fragments. The plates were then treated with ABTS substrate, which reacts with the peroxidase on the anti-DNA antibody and produces a blue colorimetric reaction. This reaction was spectrophotometricaly measured at wavelength of 405 nm and 490 nm to record background fluorescence. Background fluorescence was subtracted from the fluorescence at 490 nm and treatment groups were normalized to the vehicle control.

For Cell Death ELISA analysis, cells were plated at $2X10^5$ in 2 mls of media in 6 well plates. Cells were incubated overnight before treatment. Vehicle and TRAIL alone groups were treated with 2 μl of 50% DMSO/ddH_2O for a final concentration of 0.05%DMSO. BITC combined and BITC with TRAIL groups were pretreated with a final concentration of 5 μM BITC overnight. The following morning vehicle, and BITC groups were treated with 2 μl 0.1% BSA and TRAIL and BITC combined with TRAIL groups were treated with a final concentration of 10 ng/ml TRAIL for 6 hours. Total treatment time with BITC was 24 hours. Cells were then collected for analysis with the Cell Death ELISA assay following the manufacturer's protocol.

Cell Counting for ELISA Normalization

Cells were trypsinized and resuspended in saved media. Cells were centrifuged for 500 RPM for 5 minutes. Supernatant was discarded and pellet was resuspended in 1 ml 1X PBS. Cells were counted using trypan blue exclusion with a hemocytometer. Four fields were counted 6 times and the cell counts averaged. This procedure was repeated for three independent trials for each cell line. Percent inhibition of proliferation compared to vehicle was calculated. These values were then used to normalize the Cell Death ELISA results.

Statistical Analysis

Statistical analysis was done using a one-way ANOVA. Results were considered to be statistically significant at p value ≤ 0.05. All experiments were done independently, a minimum of 3 times.

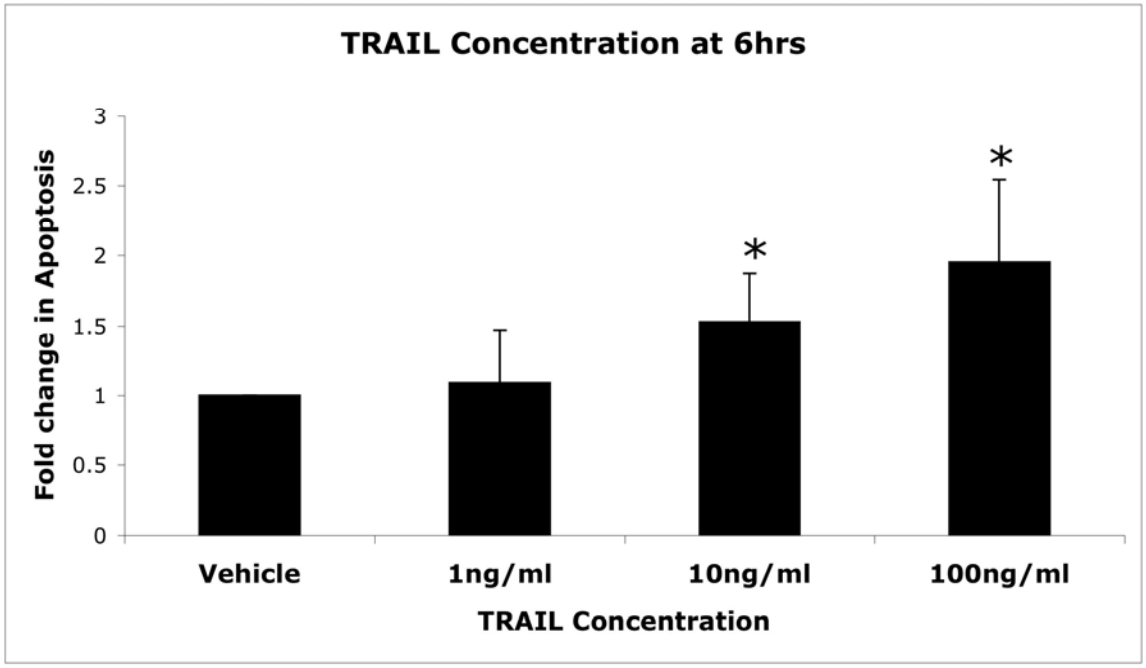
III. RESULTS

Determination of optimal effective concentration of TRAIL

Resistance to TRAIL-induced apoptosis has previously been shown in pancreatic cancer cells (49). To determine the optimal effective concentration of TRAIL needed to induce apoptosis, we first treated Panc-1 cells with increasing concentration at 0, 1, 10, and 100 ng/ml TRAIL for 6 hours (Fig. 4). TRAIL-induced apoptosis was evaluated using the Cell Death ELISA assay. Increasing amounts of apoptosis was observed with increasing concentrations of TRAIL. There was no significant increase in apoptosis with 1 ng/ml TRAIL compared to the vehicle treated cells. A significant 1.5 fold increase in apoptotic death was observed with 10 ng/ml TRAIL when compared to the vehicle alone and the 100 ng/ml TRAIL showed a 1.95 fold increase in cell death ($p < 0.05$). Since a significant increase in apoptosis compared to control was observed at 10 ng/ml TRAIL and as there was no significant difference in apoptotic death between 10 ng/ml TRAIL and 100 ng/ml TRAIL, 10 ng/ml TRAIL was chosen for all further studies.

Figure 4. Determination of optimal TRAIL concentration in Panc-1 cells. Panc-1 cells were plated $1X10^5$ cells/ml. Cells were treated with 0, 1, 10, and 100 ng/ml TRAIL for 6 hours and analyzed by Cell Death ELISA assay. The results are represented by fold increase in apoptosis compared to vehicle. Error bars represent standard deviation. * Denotes significance to $p \leq 0.05$

Figure 4. Determination of optimal TRAIL concentration in Panc-1 cells.

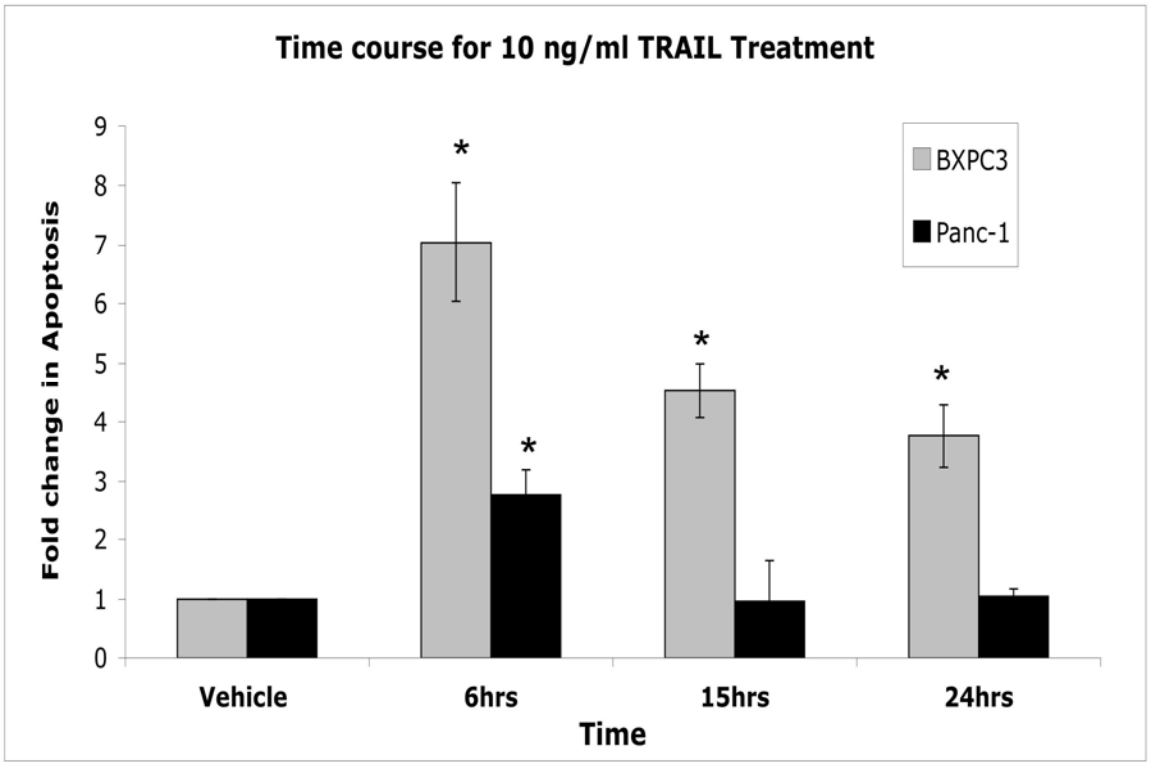


Determination of optimal treatment time with TRAIL

Next, we determined the optimum duration for TRAIL treatment. Panc-1 and BxPC3 cells were treated with 10 ng/ml TRAIL for 6, 15 and 24 hours and apoptosis was analyzed using the Cell Death ELISA assay (Fig. 5). When compared to the vehicle there was a 7.04 fold increase in cell death at 6 hours in BxPC3 cells, at 15 hours there was a 4.53 fold increase in cell death and at 24 hours there was a 3.76 fold increase in cell death. In Panc-1 cells, at 6 hours there was a 2.76 fold increase, at 15 hours there was a 0.96 fold increase in cell death, and at 24 hours there was a 1.05 fold increase in cell death. Higher levels of TRAIL-induced apoptosis were observed in wildtype K-Ras 12 BxPC3 cells, than seen in K-Ras 12 mutant Panc-1 cell line. The highest fold increase in apoptotic death (7 fold compared to the vehicle) was observed at 6 hours and this time point was chosen for all further experiments.

Figure 5. Determination of optimal treatment duration with TRAIL in BxPC3 and Panc-1 cell lines. BxPC3 and Panc-1 cells were plated at $1X10^5$ cells/ml. Cells were treated with 10 ng/ml TRAIL for 0, 6, 8, 15 and 24 hours and analyzed using Roche Cell Death ELISA. The results are represented by fold increase in apoptosis compared to vehicle. Error bars represent standard deviation. * Denotes significance to $p \leq 0.05$

Figure 5.


Time course for 10 ng/ml TRAIL Treatment
Fold change in Apoptosis
0
1
2
3
4
5
6
7
8
9
*
*
*
*
BXPC3
Panc-1
Vehicle
6hrs
15hrs
24hrs
Time

Apoptotic induction in BxPC3, MiaPaCa2, and Panc-1 cell lines

BITC is known to induce cell cycle arrest in human pancreatic cancer cells (25,26,37,39). We analyzed the effect of BITC on TRAIL-induced apoptosis in BxPC3, MiaPaCa2, and Panc-1 cells by the Cell Death ELISA assay (Fig. 6). Cells were treated with vehicle, BITC alone, TRAIL alone or BITC in combination with TRAIL. The Cell Death ELISA results indicated BITC alone caused a modest, nonsignificant increase in apoptosis in BxPC3, MiaPaCa2, and Panc-1 cell lines with a 1.5-3 fold increase in apoptosis. Baseline TRAIL sensitivity in the three cell lines was determined. BxPC3 and MiaPaCa2 cells were the sensitive to TRAIL-induced apoptosis with a 8-fold increase in apoptosis compared to vehicle. Panc-1 cells were the most resistant with only a 1.6 fold increase in apoptosis with TRAIL alone.

Inhibition of cell growth in BxPC3, MiaPaCa2, and Panc-1 cell lines

Because BITC is a known cell cycle inhibitor, the fold change in cell death was normalized to the cell number to accurately reflect the degree of apoptosis (Fig. 7, Fig. 8). Cell counts were performed to determine effects of treatment on cellular proliferation (Fig. 7). In BxPC3 cells, BITC led to a 46% reduction in cell number. TRAIL caused a 14% reduction and BITC combined with TRAIL caused a 49% reduction in cell number. In MiaPaCa2 cells, BITC led to a 34% decrease in cell number. TRAIL caused a 7% reduction and BITC combined with TRAIL caused a 54% reduction in cell number.

Figure 6. Quantification of apoptotic death in BxPC3, MiaPaCa2, and Panc-1 cell lines. BxPC3, MiaPaCa2, and Panc-1 cells were plated at $1X10^5$/ml in RPMI 1640 with 5 % FBS. Cells were treated with vehicle (V), 5 μM BITC for 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both B and T (BT). Cells were treated with 10 ng/ml TRAIL and analyzed by Cell Death ELISA. Cell Death ELISA results are represented by fold increase in apoptosis compared to vehicle. Error bars represent standard deviation. * Denotes significance to $p \leq 0.05$

Figure 6.

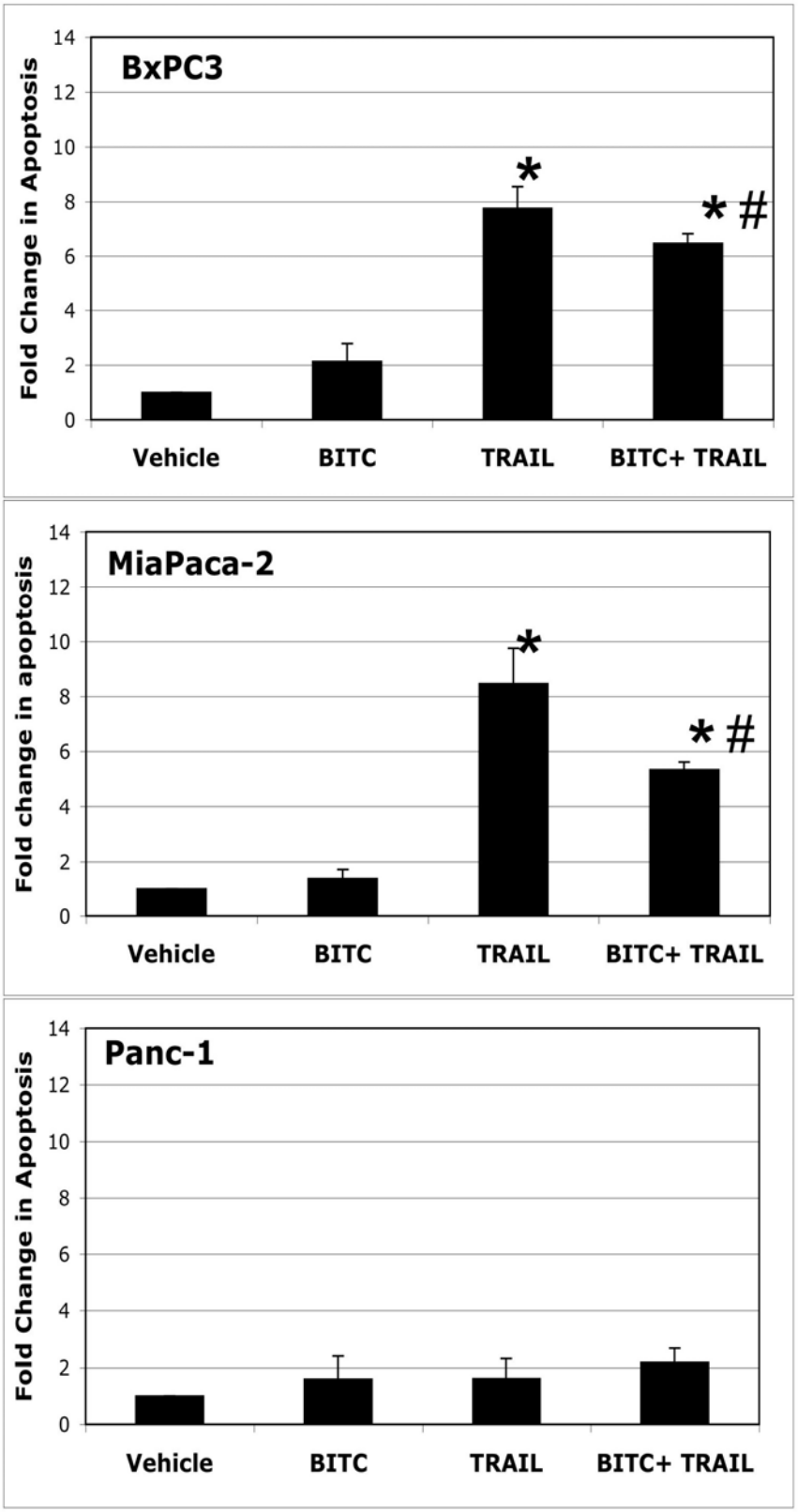

BxPC3
Fold Change in Apoptosis
14
12
10
8
6
4
2
0
*
*#
Vehicle
BITC
TRAIL
BITC+ TRAIL
MiaPaca-2
Fold change in apoptosis
14
12
10
8
6
4
2
0
*
*#
Vehicle
BITC
TRAIL
BITC+ TRAIL
Panc-1
Fold Change in Apoptosis
14
12
10
8
6
4
2
0
Vehicle
BITC
TRAIL
BITC+ TRAIL

Figure 7. Inhibition of cell growth in BxPC3, MiaPaCa2, and Panc-1 cell lines. BxPC3, MiaPaCa2, and Panc-1 cells were plated at $1X10^5$/ml in RPMI 1640 with 5 % FBS. Cells were treated with vehicle (V), 5 μM BITC for 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both B and T (BT). Cell counts were performed using trypan blue for a minimum of four fields, six times and averaged to determine the effects of treatments on cell growth. * Denotes significance to $p \leq 0.05$. # Denotes significance to $p \leq 0.05$ between T and BT.

Figure 7.

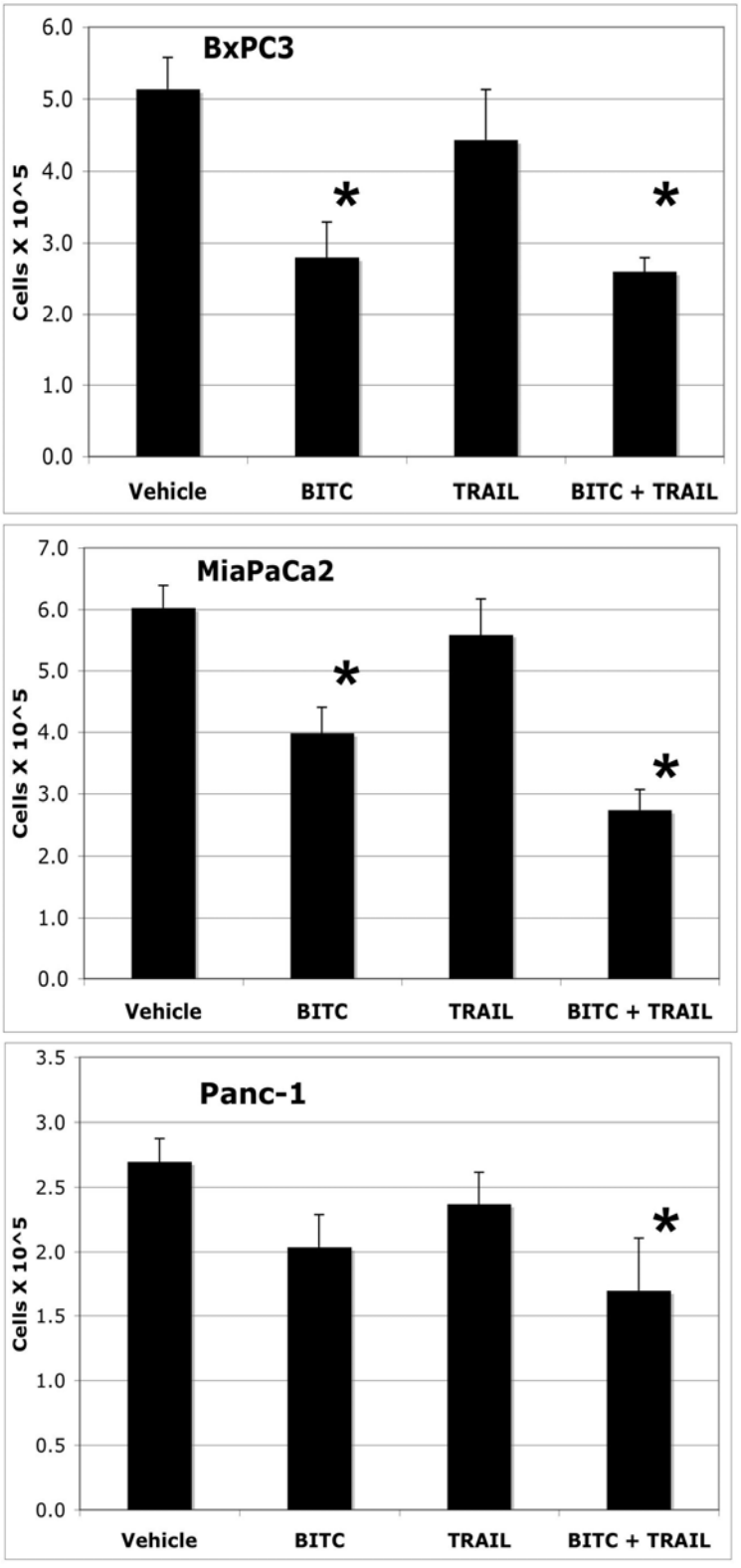
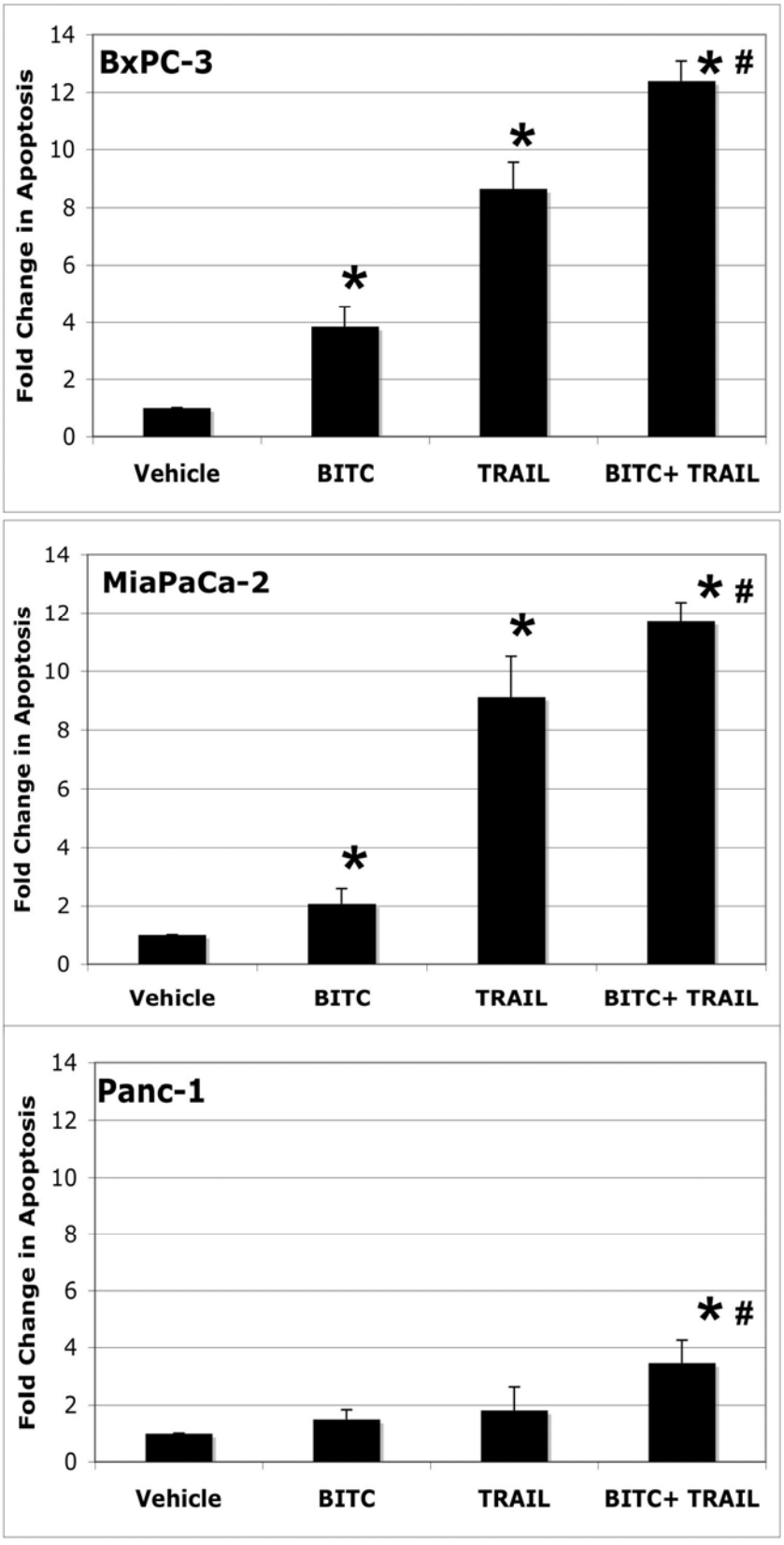

BxPC3
Cells X 10^5
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
Vehicle
BITC
TRAIL
BITC + TRAIL
MiaPaCa2
Cells X 10^5
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
Vehicle
BITC
TRAIL
BITC + TRAIL
Panc-1
Cells X 10^5
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Vehicle
BITC
TRAIL
BITC + TRAIL

Figure 8. Normalized Apoptotic Induction in BxPC3, MiaPaCa2, and Panc-1 cell lines. BxPC3, MiaPaCa2, and Panc-1 cells were plated at $1X10^5$/ml in RPMI 1640 with 5 % FBS. Cells were treated with vehicle (V), 5 μM BITC for 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both B and T (BT). In Fig. 1a and b, cells were collected and analyzed using Roche Cell Death ELISA following the manufacturer's recommended protocol. The results are represented by fold increase in apoptosis compared to vehicle and represent a minimum of 3 independent trials. These data represent Cell Death ELISA results after normalization to cell count. Percent decrease in cell numbers compared to vehicle was calculated and used to normalize the Cell Death ELISA results. Error bars represent standard deviation. * Denotes significance to $p \leq 0.05$ # Denotes significance to $p \leq 0.05$ between T and BT.

Figure 8.


BxPC-3
Fold Change in Apoptosis
14
12
10
8
6
4
2
0
Vehicle
BITC
TRAIL
BITC+ TRAIL
* #
MiaPaCa-2
Fold Change in Apoptosis
Vehicle
BITC
TRAIL
BITC+ TRAIL
* #
Panc-1
Fold Change in Apoptosis
Vehicle
BITC
TRAIL
BITC+ TRAIL
* #

In Panc-1 cells, BITC led to a 25% decrease, whereas TRAIL alone caused a 12% decrease and BITC combined with TRAIL caused a 37% decrease in cell number (Fig. 6).

Normalized Apoptotic Induction in BxPC3, MiaPaCa2, and Panc-1 cell lines

Higher amounts of cell death were observed in all cells treated with TRAIL alone when compared with TRAIL combined with BITC. BITC is a known cell cycle inhibitor, and believed to arrest cells in the G2/M phase. To address inconsistencies that may arise during Cell Death ELISA assay due to a decrease in cell numbers, we normalized the Cell Death ELISA assay results to cell number to accurately reflect the degree of apoptosis (Fig. 8). With cell number taken into consideration, the normalized Cell Death ELISA showed a 3.84 fold increase in apoptosis in BxPC3 upon treatment with BITC alone, a 8.65 fold increase was observed with TRAIL alone, and a 12.39 fold increase was seen in BxPC3 cells treated with BITC combined with TRAIL compared to vehicle. MiaPaCa2 showed a 2.08 fold increase in apoptosis upon treatment with BITC, a 9.12 fold increase with TRAIL alone, and a 11.72 fold increase with BITC combined with TRAIL compared to vehicle. Panc-1 cells showed a 1.49 fold increase in apoptosis upon treatment with BITC, a 1.82 fold increase with TRAIL alone, and a 3.45 fold increase with BITC combined with TRAIL compared to vehicle. BITC combined with TRAIL produced a significant increase in apoptotic cell death compared to TRAIL alone in all three cell lines.

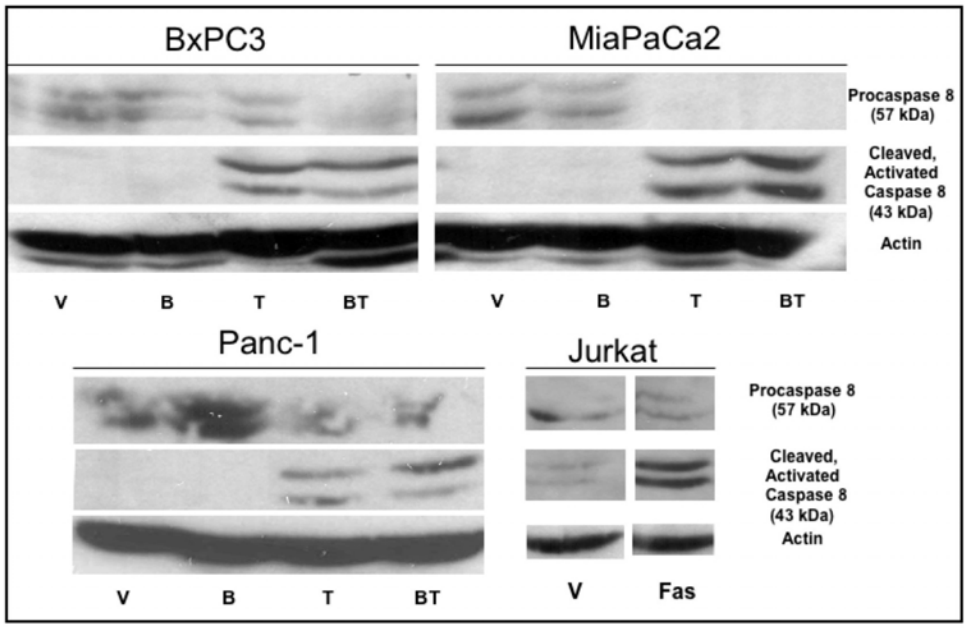
Activation of caspase 8 in BxPC3, MiaPaCa2, and Panc-1 cell lines

Western blot analysis was performed to determine the apoptotic pathways involved in the BITC and TRAIL-induced apoptosis in pancreatic adenocarcinoma cells. BxPC3, MiaPaCa2, and Panc-1 cells were treated with vehicle, BITC, TRAIL, or BITC combined with TRAIL and examined for activation of extrinsic cell death pathway as suggested by caspase 8 cleavage (Fig. 9)(35,46,54). In BxPC3 cells, vehicle alone had no effect on caspase 8. BITC alone led to a 6.84 fold decrease in the 57 kDa proform and a 0.5 fold increase in the 43 kDa cleaved caspase 8 compared to vehicle. Treatment with TRAIL alone showed a 5.15 fold increase in cleavage of the inactive, 57 kDa proform of caspase 8 and produced a 3.27 fold decrease in proform levels. A combination of BITC with TRAIL showed further loss of the intact proform with a 3.66 fold decrease compared to vehicle while cleaved 43 kDa form levels remained similar. In MiaPaCa2 cells, there was no cleavage of caspase 8 with vehicle. BITC alone led to a 1.13 fold decrease in caspase 8 proform and a 0.70 fold increase in cleaved caspase 8 levels compared to vehicle. TRAIL alone produced a 9.28 fold decrease of the caspase 8 proform with a 9.18 fold increase in caspase 8 cleavage. Combination of BITC with TRAIL caused led to a 3.55 fold decrease in caspase 8 proform and a 20.98 fold increase in cleaved caspase 8 levels. In Panc-1 cells, BITC alone produced a 0.83 fold decrease in caspase 8 proform levels and a 0.46 fold increase in cleaved caspase 8. TRAIL alone produced a 1.12 fold decrease in proform levels and a 3.52 increase in cleaved caspase 9. BITC combined with TRAIL produced a 3.14 fold decrease in caspase 8 levels and a 5.26 increase in cleaved caspase 8 levels compared to vehicle. BITC increased cleavage of procaspase 8 in BxPC3 and Panc-1 cell lines compared to vehicle. There were greater

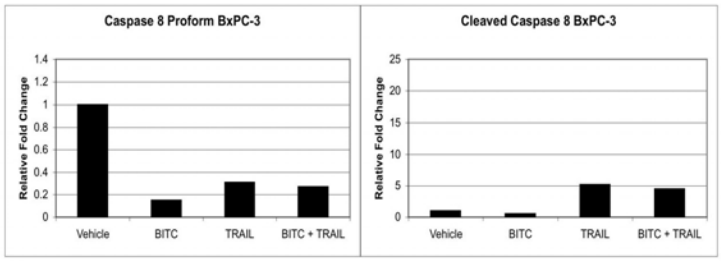
Figure 9. Caspase 8 activation in human pancreatic cancer cell lines. Activation of caspase 8 in BxPC3, MiaPaCa2 and Panc-1 cell lines upon treatment with vehicle (V), 5 μM BITC for 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both 5 μM BITC and 10 ng/ml TRAIL (BT). Total treatment time was 24 hours. Whole cell lysates were collected and separated by SDS-PAGE, and analyzed by Western blotting described in the Methods and Materials. The blot was probed with active caspase 8 and reprobed with actin to ensure equal protein loading.

This graph illustrates relative fold change in caspase 8 proform and cleaved caspase 8 in A. BxPC3, B. MiaPaCa2 and C. Panc-1 cell lines upon treatment with vehicle, BITC alone, TRAIL alone, or BITC combined with TRAIL. Fold change was determined using NIH image analysis of a Western blot for caspase 8 normalized to the vehicle. NIH image was developed by NIH and can be found at http://rsb.info.nih.gov/nih-image/.

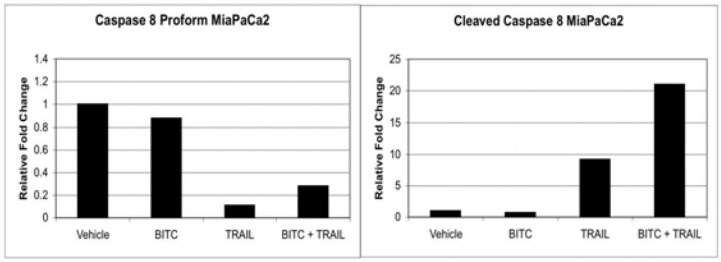
Figure 9.


BxPC3
MiaPaCa2
Procaspase 8 (57 kDa)
Cleaved, Activated Caspase 8 (43 kDa)
Actin
V
B
T
BT
V
B
T
BT
Panc-1
Jurkat
Procaspase 8 (57 kDa)
Cleaved, Activated Caspase 8 (43 kDa)
Actin
V
B
T
BT
V
Fas

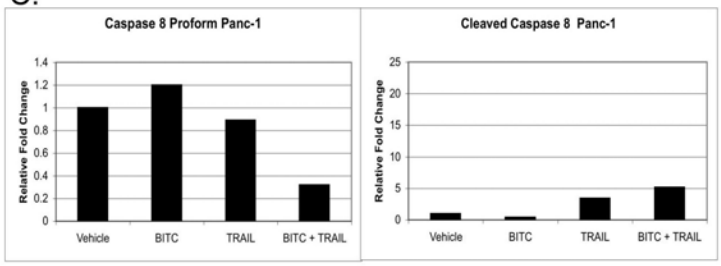
A.


Caspase 8 Proform BxPC-3
Relative Fold Change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL
Cleaved Caspase 8 BxPC-3
Relative Fold Change
25
20
15
10
5
0
Vehicle
BITC
TRAIL
BITC + TRAIL

B.


Caspase 8 Proform MiaPaCa2
Relative Fold Change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL
Cleaved Caspase 8 MiaPaCa2
Relative Fold Change
25
20
15
10
5
0
Vehicle
BITC
TRAIL
BITC + TRAIL

C.


Caspase 8 Proform Panc-1
Relative Fold Change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL
Cleaved Caspase 8 Panc-1
Relative Fold Change
25
20
15
10
5
0
Vehicle
BITC
TRAIL
BITC + TRAIL

levels of cleaved caspase 8 in MiaPaCa2 and Panc-1 cells with combined treatment compared to TRAIL alone.

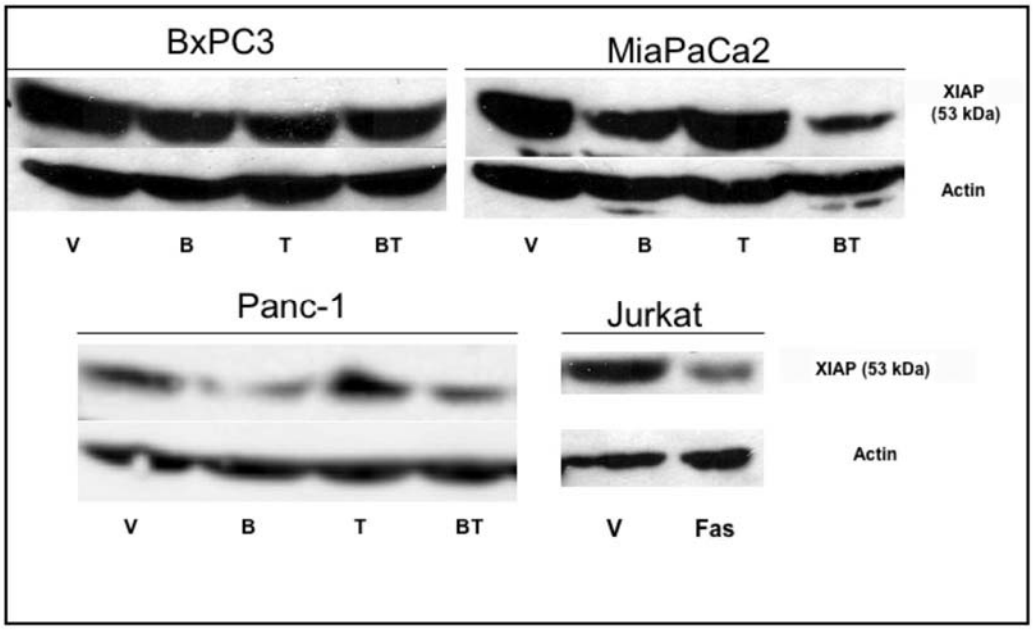
XIAP cleavage in BxPC3, MiaPaCa2, and Panc-1 cell lines

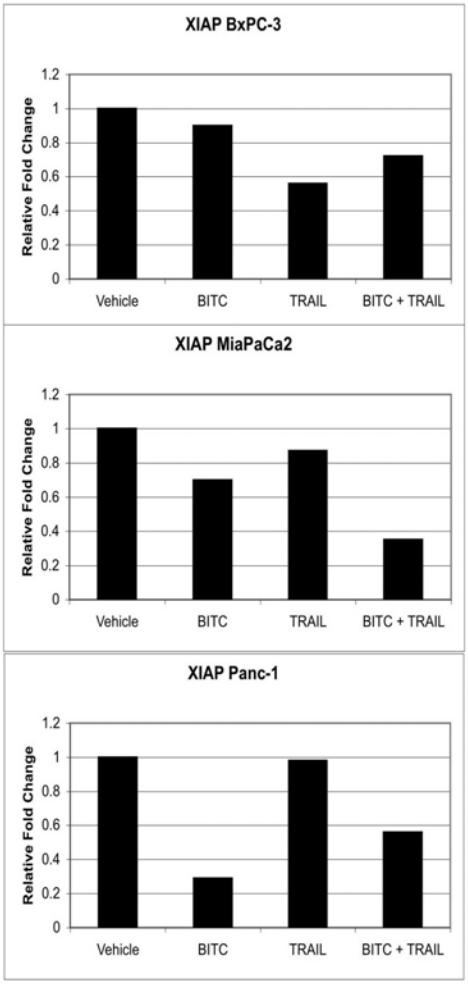
X-linked inhibitor of apoptosis (XIAP) is a 53 kDa prosurvival protein and a direct target of caspase 8. Reduction of intact XIAP was determined to further confirm caspase 8 activation. NIH image was used to determine changes in levels of XIAP in BxPC3, MiaPaCa2 and Panc-1 cells upon treatment with vehicle, BITC alone, TRAIL alone and BITC combined with TRAIL (Fig. 10). In BxPC3 cells, BITC alone produced a 1.12 fold decrease in XIAP compared to vehicle. TRAIL alone produced a 1.77 fold decrease and BITC combined with TRAIL produced a 1.38 fold decrease in intact XIAP levels. In MiaPaCa2 cells, BITC alone produced a 1.42 fold decrease in XIAP. TRAIL alone produced a 1.15 fold decrease and BITC combined with TRAIL 2.89 fold decrease in intact XIAP levels. In Panc-1 cells, BITC alone produced a 3.45 fold decrease in XIAP. TRAIL alone produced a 1.02 fold decrease and BITC combined with TRAIL produced a 1.79 fold decrease in intact XIAP levels. A 1.99 fold decrease was observed in Jurkat cells treated with anti-Fas antibody, which were used as a positive control for XIAP cleavage. MiaPaCa2 and Panc-1 cell lines show increased XIAP cleavage upon with combined treatment with BITC and TRAIL compared to TRAIL alone.

Figure 10. XIAP cleavage in BxPC3, MiaPaca2, and Panc-1 cell lines. XIAP cleavage in BxPC3, MiaPaCa2 and Panc-1 cell lines upon treatment with vehicle (V), 5 μM BITC for 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both BITC and TRAIL. Total treatment time was 24 hours. Whole cell lysates were collected and separated through SDS-PAGE, and Western blotting was performed as described in the Methods and Materials. The blot was probed with XIAP antibody and reprobed with pan actin to ensure equal loading.

This graph represents fold change in XIAP protein levels in BxPC3, MiaPaCa2, and Panc-1 cell lines upon treatment with vehicle, BITC alone, TRAIL alone, and BITC combined with TRAIL. Fold change was determined using NIH image analysis of a Western blot for XIAP normalized to the vehicle. NIH image was developed by NIH and can be found at http://rsb.info.nih.gov/nih-image/.

Figure 10.


BxPC3
MiaPaCa2
XIAP (53 kDa)
Actin
V
B
T
BT
V
B
T
BT
Panc-1
Jurkat
XIAP (53 kDa)
Actin
V
B
T
BT
V
Fas


XIAP BxPC-3
Relative Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL
XIAP MiaPaCa2
Relative Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL
XIAP Panc-1
Relative Fold Change
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL

Bid cleavage in BxPC3, MiaPaCa2 and Panc-1 cell lines

The death receptor pathway is known to activate caspase 8 and caspase 3 alone or trigger the intrinsic pathway via cleavage and activation of 22 kDa Bid (t-Bid) (35). BxPC3, MiaPaCa2, and Panc-1 cells treated with vehicle, BITC alone, TRAIL alone, or BITC combined with TRAIL were examined for Bid truncation (Fig. 11). NIH image was used to analyze levels of Bid protein in BxPC3, MiaPaCa2, and Panc-1 cells upon treatment with vehicle, BITC alone, TRAIL alone and BITC combined with TRAIL. In BxPC3 cells, BITC alone led to a 0.74 fold decrease in intact Bid compared to vehicle. TRAIL alone led to a 7.08 fold change and BITC combined with TRAIL led to a 8.91 fold decrease in intact Bid levels. In MiaPaCa2 cells, BITC alone produced a 1.28 fold decrease in Bid. TRAIL alone produced a 18.32 fold decrease and BITC combined with TRAIL produced a 7.54 fold decrease in intact Bid levels. In Panc-1 cells, BITC alone led to a 1.38 fold decrease in Bid levels. TRAIL alone led to a 1.88 fold decrease and BITC combined with TRAIL led to a 3.14 fold change in intact Bid levels. A 2.59 fold decrease was observed in Jurkat cells treated with anti-Fas antibody, which were used as a positive control for Bid cleavage. In Panc-1 cell lines, increased Bid cleavage was observed upon treatment with BITC combined with TRAIL compared to TRAIL alone.

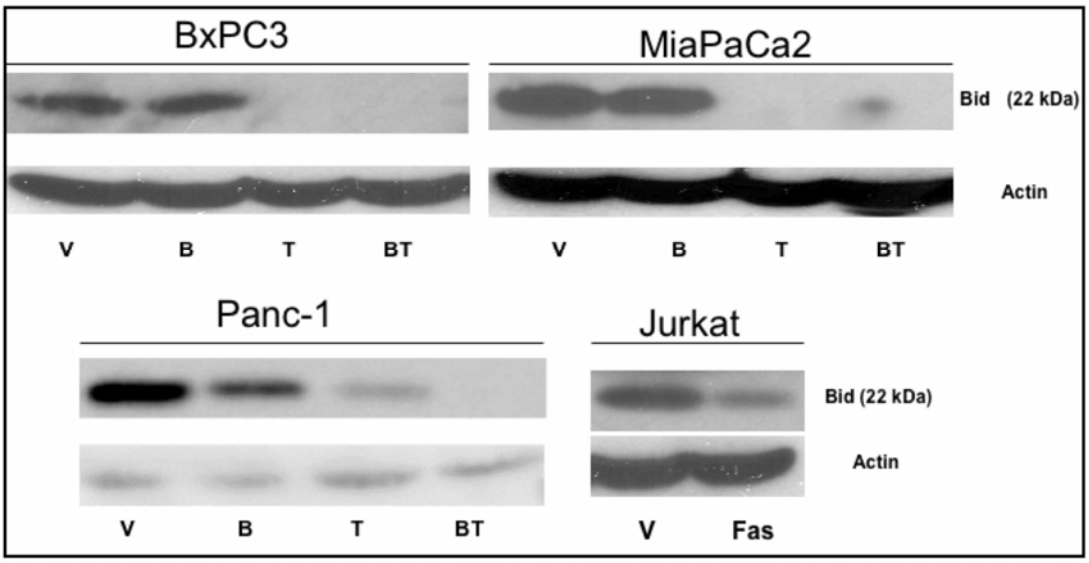
Caspase 9 activation BxPC3, MiaPaCa2, and Panc-1 cell lines

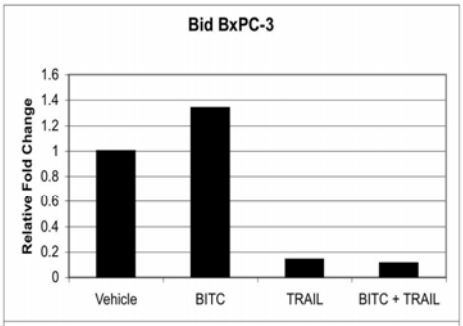
Truncation of Bid has been shown to induce the intrinsic cell death pathway (35,56). We sought to determine whether TRAIL combined with BITC could activate the intrinsic, mitochondrial cell death pathway. BxPC3, MiaPaCa2 and Panc-1 cells were

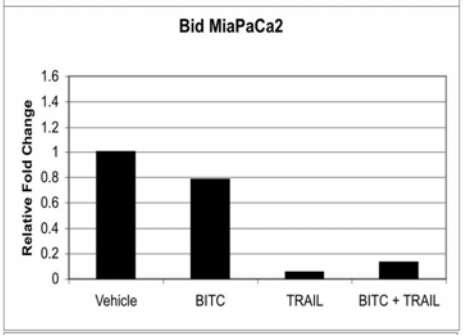
Figure 11. TRAIL-induced loss of intact Bid protein. Bid cleavage in BxPC3, MiaPaCa2 and Panc-1 cell lines upon treatment with vehicle (V), 5 μM BITC 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both B and T. Total treatment time was 24 hours. Whole cell lysates were collected and separated through SDS-PAGE, and Western blotting was performed as described in the Methods and Materials. The blot was probed with bid antibody and reprobed with pan actin used as a loading control

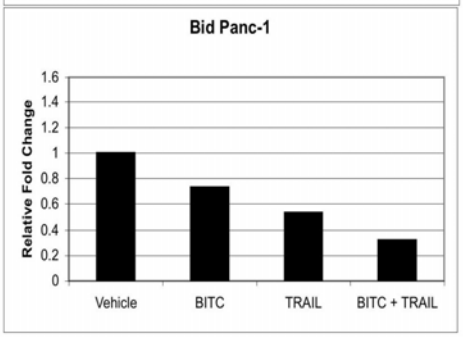
This graph illustrates relative fold change in intact Bid protein levels in BxPC3, MiaPaCa2 and Panc-1 cell lines upon treatment with vehicle, BITC alone, TRAIL alone, or BITC combined with TRAIL. Fold change was determined using NIH image analysis of a Western blot for Bid normalized to the vehicle. NIH image was developed by NIH and can be found at http://rsb.info.nih.gov/nih-image/.

Figure 11.


BxPC3
MiaPaCa2
Bid (22 kDa)
Actin
V
B
T
BT
V
B
T
BT
Panc-1
Jurkat
Bid (22 kDa)
Actin
V
B
T
BT
V
Fas


Bid BxPC-3
Relative Fold Change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL


Bid MiaPaCa2
Relative Fold Change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL

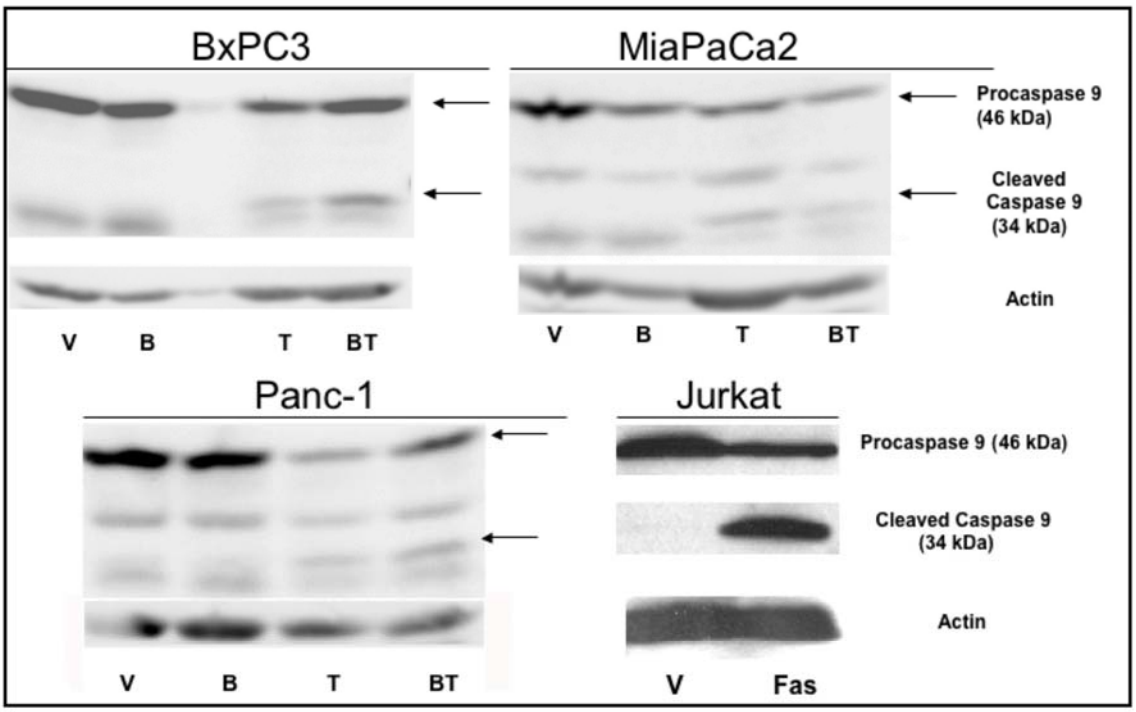

Bid Panc-1
Relative Fold Change
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
BITC
TRAIL
BITC + TRAIL

examined for the activation of caspase 9, initiator caspase for the intrinsic cell death pathway (Fig. 12)(35). NIH image was used to determine the effects of vehicle, BITC alone, TRAIL alone and BITC combined with TRAIL on levels of the 46 kDa caspase 9 proform and the 34 kDa cleaved form in BxPC3, MiaPaCa2 and Panc-1 cells. In BxPC3 cells, BITC alone led to a 1.03 fold decrease in caspase 9 proform compared to vehicle. TRAIL alone led to a 1.97 fold decrease and BITC combined with TRAIL led to a 1.08 fold decrease in caspase 9 proform levels. BITC produced a 1.53 fold change in cleaved caspase 9 levels. TRAIL alone produced a 4.26 fold change and BITC combined with TRAIL produced a 9.28 fold increase in cleaved caspase 9 levels. In MiaPaCa2 cells, BITC alone led to a 1.47 fold decrease in caspase 9 proform levels. TRAIL alone led to a 1.81 fold decrease and BITC combined with TRAIL led to a 2.11 fold decrease in levels of caspase 9 proform. BITC produced 0.13 fold change in cleaved caspase 9. TRAIL alone produced a 0.5 fold change and BITC combined with TRAIL produced a 1.5 fold increase in cleaved caspase 9. In Panc-1 cells, BITC led to a 1.61 fold decrease in caspase 9 proform levels compared to vehicle. TRAIL alone led to a 2.02 fold decrease and BITC combined with TRAIL led to a 2.17 fold change in caspase 9 proform levels. BITC alone produced a 0.66 fold increase in cleaved caspase 9 compared to vehicle. TRAIL alone produced a 1.26 fold increase and BITC combined with TRAIL produced 2.93 fold increase in cleaved caspase 9 levels. A 1.53 fold decrease in proform and 4.23 fold increase in cleaved caspase 9 was observed in Jurkat cells treated with anti-Fas antibody, which was used as a positive control for caspase 9 cleavage. In BxPC3, MiaPaCa2, and Panc-1 cell lines, a decrease in proform and increase in cleaved caspase 9 was observed upon treatment with TRAIL alone. Further increase in

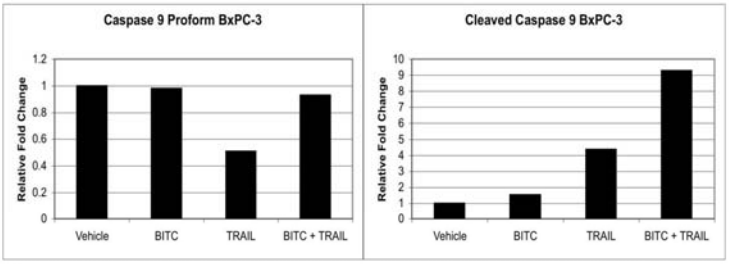
Figure 12. Caspase 9 activation in BxPC3, MiaPaCa2, and Panc-1 cell lines. Activation of caspase 9 in BxPC3, MiaPaCa2 and Panc-1 cell lines upon treatment with vehicle (V), 5 μM BITC 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both BITC and TRAIL Total treatment time was 24 hours. Whole cell lysates were collected and separated through SDS-PAGE, and Western blotting was performed as described in the Methods and Materials. Arrows indicated procaspase 9 and the active fragment. The blot was probed with active caspase 9 antibody and reprobed with pan actin used as a loading control.

This graph illustrates relative fold change in caspase 9 proform and cleaved caspase 9 protein levels in A. BxPC3, B. MiaPaCa2 and C. Panc-1 cell lines upon treatment with vehicle, BITC alone, TRAIL alone, or BITC combined with TRAIL. Fold change was determined using NIH image analysis of a Western blot for caspase 9 normalized to the vehicle. NIH image was developed by NIH and can be found at http://rsb.info.nih.gov/nih-image/.

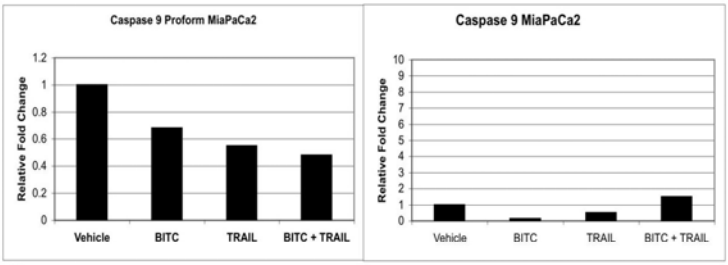
Figure 12.



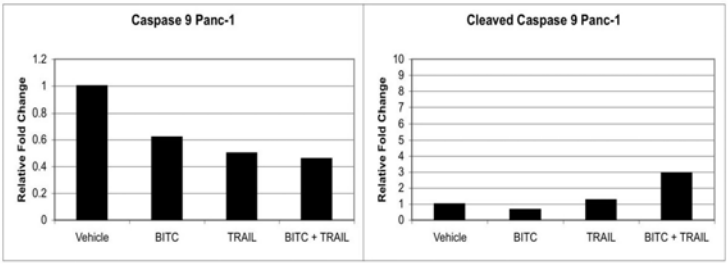
A.



B.



C.



caspase 9 cleavage was observed upon treatment with BITC combined with TRAIL in all three cell lines.

Activation of caspase 3 in BxPC3, MiaPaCa2, and Panc-1 cell lines

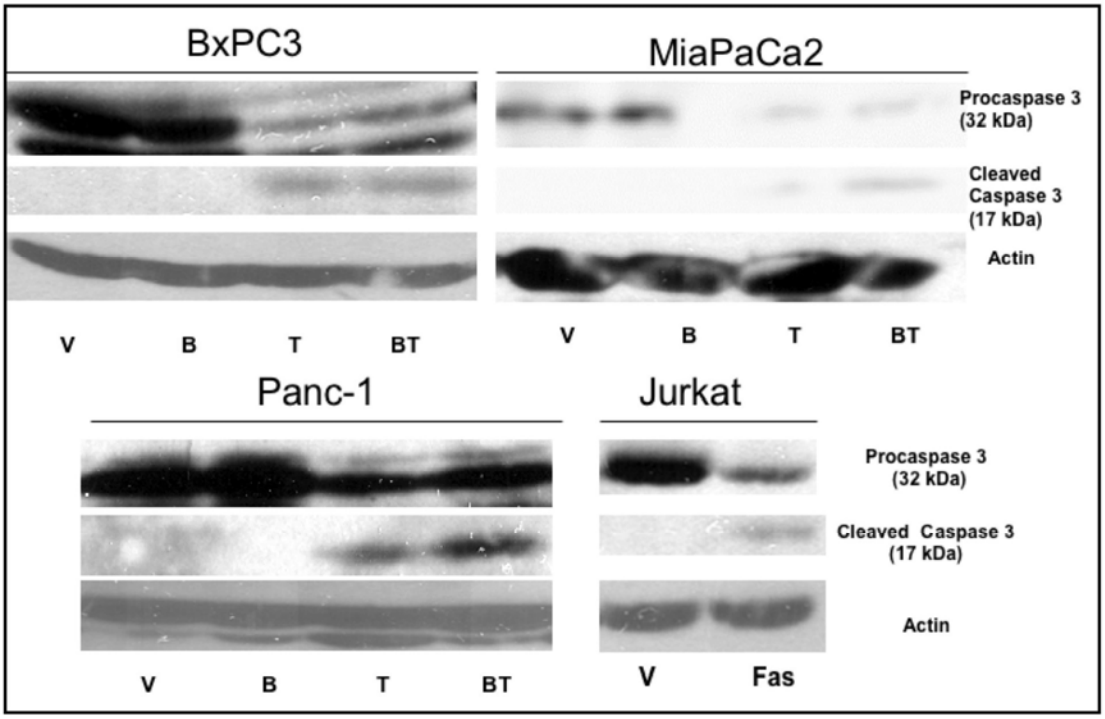
Because both the extrinsic and intrinsic cell death pathways trigger effector caspase cleavage, we analyzed for the effect of BITC and TRAIL on the major effector caspase, caspase 3 (Fig. 13). The 32 kDa caspase 3 proform and the 17 kDa cleaved form were analyzed using NIH image to quantitate changes in protein level upon treatment with vehicle, BITC alone, TRAIL alone and BITC combined with TRAIL in BxPC3, MiaPaCa2 and Panc-1 cells. In BxPC3 cells, BITC alone led to a fold change in caspase 3 proform compared to vehicle. TRAIL alone led to a 3.57 fold decrease and BITC combined with TRAIL led to a 2.37 fold decreases in caspase 3 proform. BITC alone produced to a 0.91 fold increase in cleaved caspase 3. TRAIL alone produced 5.43 fold increase and BITC combined with TRAIL produced a 9.83 fold increase in cleaved caspase 3 levels. In MiaPaCa2 cells, BITC alone led to a 1.39 fold decrease in caspase 3 proform. TRAIL alone led to a 7.20 fold decrease and BITC combined with TRAIL led to a 5.25 fold decrease in caspase 3 proform. BITC alone produces a 2.11 fold increase in cleaved caspase 3. TRAIL alone produced a 2.11 fold increase and BITC combined with TRAIL produced a 8.21 fold increase in cleaved caspase 3. In Panc-1 cells, BITC alone produced a 1.47 fold decrease fold decrease in caspase 3 proform. TRAIL alone led to a 1.65 fold decrease and BITC combined with TRAIL led to a 1.23 fold decrease in caspase 3 proform. BITC alone produced a 0.34 fold increase in cleaved caspase 3. TRAIL alone produced a 1.87 fold increase and a 2.92 fold increase in cleaved caspase 3 levels. A 4.80 fold decrease in proform and a 5.85 fold increase in cleaved caspase 3

Figure 13. Caspase 3 activation in BxPC3, MiaPaCa2, and Panc-1 cells.

Cleavage of effector caspase 3 in BxPC3, MiaPaCa2 and Panc-1 cell lines upon treatment with vehicle (V), 5 μM BITC 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both B and T. Total treatment time was 24 hours. Whole cell lysates were collected and separated through SDS-PAGE, and Western blotting was performed as described in the Methods and Materials. The same blot was probed with pan actin used as a loading control

This graph illustrates relative fold change in caspase 3 proform and cleaved caspase 3 protein levels in A. BxPC3, B. MiaPaCa2 and C. Panc-1 cell lines upon treatment with vehicle, BITC alone, TRAIL alone, or BITC combined with TRAIL. Fold change was determined using NIH image analysis of a Western blot for caspase 3 normalized to the vehicle. NIH image was developed by NIH and can be found at http://rsb.info.nih.gov/nih-image/.

Figure 13.



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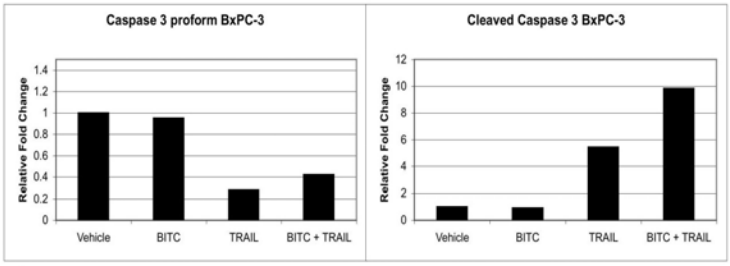

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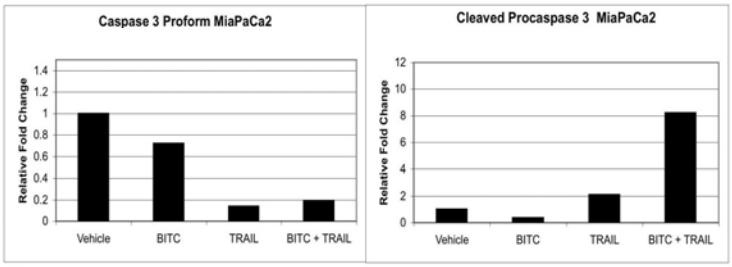

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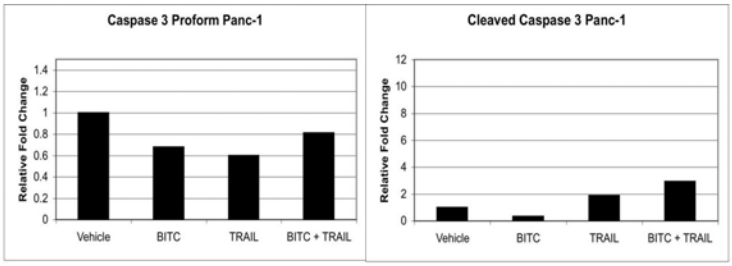


was observed in Jurkat cells treated with an anti-Fas antibody, which was used as a positive control for caspase 3 cleavage. In BxPC3, MiaPaCa2, and Panc-1 cell lines, combined treatment with BITC and TRAIL increased cleaved caspase 3 levels compared to TRAIL alone.

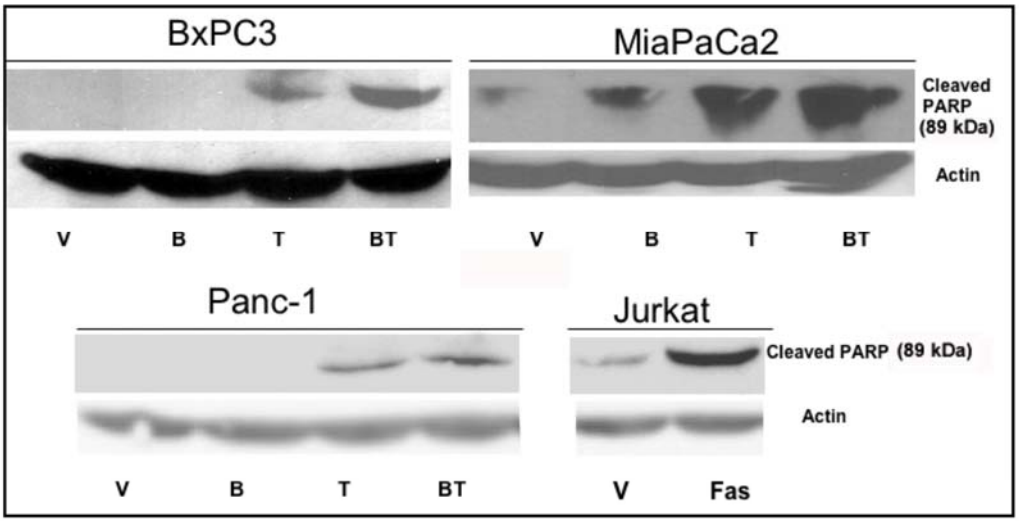
PARP cleavage in BxPC3, MiaPaCa2, and Panc-1 cell lines

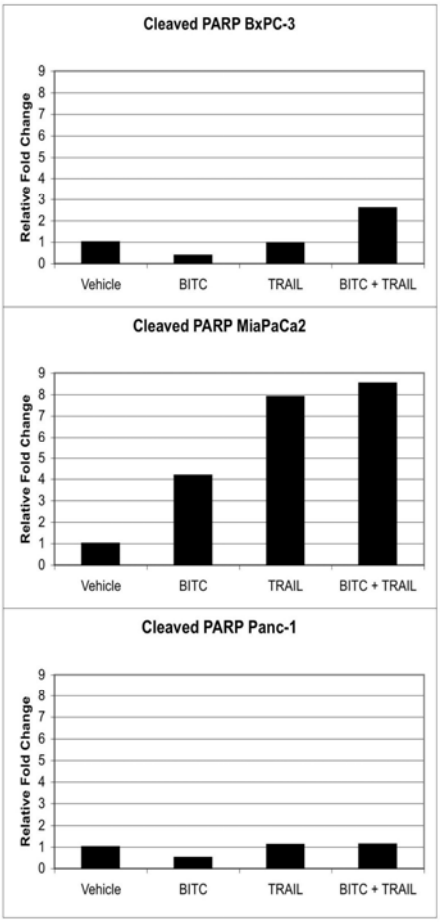
To confirm the activation of caspase 3 we examined PARP, a direct specific substrate of activated caspase 3 (Fig 14). NIH image was used to quantify changes in the 89 kDa cleaved PARP levels upon treatment with vehicle, BITC alone, TRAIL alone and BITC combined with TRAIL in BxPC3, MiaPaCa2 and Panc-1 cells. In BxPC3 cells, BITC alone produced a 0.38 fold increase in cleaved PARP. TRAIL alone produced a 0.96 fold increase and BITC combined with TRAIL produced a 2.58 fold increase in cleaved PARP compared to vehicle. In MiaPaCa2 cells, BITC alone led to a 4.17 fold increase in cleaved PARP. TRAIL alone produced a 7.90 fold increase and BITC combined with TRAIL produced a 8.52 fold increase in cleaved PARP. In Panc-1 cells, BITC alone produced a 0.50 fold increase in cleaved PARP. TRAIL alone produced a 1.09 fold increase and BITC combined with TRAIL produce 1.12 fold increase fold increase in PARP cleavage. A 1.75 fold increase in cleaved PARP was observed in Jurkat cells treated with a Fas receptor inducing antibody, which was used as a positive control for PARP cleavage. In BxPC3 and MiaPaCa2 there was increased PARP cleavage upon treatment with BITC combined with TRAIL compared to TRAIL alone. A modest increase in cleaved PARP levels was observed in Panc-1 cells treated with BITC combined with TRAIL versus TRAIL alone.

Figure 14. PARP cleavage in BxPC3, MiaPaCa2, and Panc-1 cells. PARP cleavage and inactivation in BxPC3, MiaPaCa2 and Panc-1 cell lines upon treatment with vehicle (V), 5 μM BITC 24 hours (B), 10 ng/ml TRAIL (T) for 6 hours or both B and T. Total treatment time was 24 hours. Whole cell lysates were collected and separated through SDS-PAGE, and Western blotting was performed as described in the Methods and Materials. The blot was probed with PARP antibody and reprobed with pan actin to ensure equal loading.

This graph illustrates relative fold change in cleaved PARP protein levels in BxPC3, MiaPaCa2 and Panc-1 cell lines upon treatment with vehicle, BITC alone, TRAIL alone, or BITC combined with TRAIL. Fold change was determined using NIH image analysis of a Western blot for PARP normalized to the vehicle. NIH image was developed by NIH and can be found at http://rsb.info.nih.gov/nih-image/.

Figure 14.


BxPC3
MiaPaCa2
Cleaved PARP (89 kDa)
Actin
V
B
T
BT
V
B
T
BT
Panc-1
Jurkat
Cleaved PARP (89 kDa)
Actin
V
B
T
BT
V
Fas


Cleaved PARP BxPC-3
Relative Fold Change
0
1
2
3
4
5
6
7
8
9
Vehicle
BITC
TRAIL
BITC + TRAIL
Cleaved PARP MiaPaCa2
Relative Fold Change
Vehicle
BITC
TRAIL
BITC + TRAIL
Cleaved PARP Panc-1
Relative Fold Change
Vehicle
BITC
TRAIL
BITC + TRAIL

IV. DISCUSSION

Pancreatic cancer is a one of the leading causes of death in the United States. Chemotherapeutic resistance and high mortality in pancreatic cancer are associated with point mutations within codon 12 of K-Ras (61,69). K-Ras regulates cellular proliferation in most cell types and mutations within codon 12 disrupt the normal on-off cycling, leading to constitutive signaling and proliferation (19). This study determined whether the cell cycle inhibitor, BITC, could sensitize pancreatic adenocarcinomas with mutated K-Ras to TRAIL-induced apoptosis.

Determination of optimal TRAIL dosage and treatment period

The Cell Death ELISA assay was used to quantitate apoptotic death in the pancreatic adenocarcinoma cell lines. We determined that 10 ng/ml TRAIL for 6 hours was the optimal treatment to induce apoptosis in Panc-1 cells. The time course ELISA interestingly showed a gradual decline in apoptosis from 6 hours to 24 hours (Fig. 4). Previous studies in our lab have shown that cells in culture can undergo secondary necrosis with extended treatment periods with cell death inducers (56). The observed decrease in cell death is likely a result of secondary necrosis which was not detected because the Cell Death ELISA assay can exclude necrotic death and specifically measure apoptotic death. K-Ras 12 wildtype BxPC3 cells were very sensitive to TRAIL-induced

apoptosis compared to resistant codon 12 K-Ras mutated Panc-1 cells. This may be attributed to their K-Ras mutations, in that BxPC3 cells are K-Ras 12 WT and Panc-1 cells are K-Ras 12 mutant.

Apoptotic effects of BITC and TRAIL

We next observed the effect of BITC and TRAIL in BxPC3, MiaPaCa2, and Panc-1 cells. In BxPC3, MiaPaCa2, and Panc-1 cell lines, BITC alone produced a minimum amount of apoptosis. This result is unsurprising as we intentionally treated with low doses of BITC to minimize apoptotic death from BITC alone. BITC is being used to sensitize the cells to TRAIL, which is tumor preferential unlike BITC. TRAIL induced a greater fold increase of apoptotic death as indicated by Cell Death ELISA in BxPC3 and MiaPaCa2 cells than in Panc-1 cells. The high levels of apoptosis in K-Ras 12 gly→cys, MiaPaCa2 cells and the low levels of apoptosis in K-Ras 12 gly→asp Panc-1 cells illustrates that the type of K-Ras 12 mutation may be crucial to the degree of TRAIL resistance as Panc-1 cells (K-Ras 12 gly→asp) are more resistant than MiaPaCa2 cells (K-Ras 12 gly→cys). In Panc-1 cells, between TRAIL alone and BITC with TRAIL, there is an increase in apoptosis. However, combined treatment of BITC and TRAIL, appears to decrease apoptosis in BxPC3 and MiaPaCa2 cells, which contradicts increased cleavage of key apoptotic proteins observed in Western blot analysis. Therefore, further analysis was done on the inhibitory effects of BITC and TRAIL on cell growth to determine if the observed decrease was due to a decrease in cell numbers.

Inhibitory effects of BITC and TRAIL on cell growth

Initial Cell Death ELISA results showed that BITC combined with TRAIL decreased apoptotic death compared to TRAIL alone. However, observations in cell culture and with Western blot analysis of key apoptotic proteins led us to further explore these results. Upon further analysis, the decrease in apoptosis seen in cells treated with both BITC and TRAIL could be a result of BITC halting cell division. Previous studies have reported BITC functions through cell cycle arresting in pancreatic adenocarcinoma cell lines (25,36-40). BITC inhibited cell growth in BxPC3 and MiaPaCa2 cells nearly 1.5 times more than in Panc-1 cells. This could be related to the growth rate of these cells. BxPC3 cells have a doubling rate of 24 hours, MiaPaCa2 cells have a doubling rate of 19 hours, and Panc-1 cells have a doubling rate of 52 hours (70,71). The cell cycle inhibiting effects of BITC were not fully apparent in Panc-1 cells, possibly due to a slower division rate. The cells that divided more rapidly such as the BxPC3 and MiaPaCa2 cells were more prone to BITC's effects. Although initially seeded at equal cell numbers, groups treated with BITC had less cells compared to groups treated with vehicle or TRAIL alone. Since fewer cells were present to undergo apoptosis in the BITC combined with TRAIL treatment group as compared to TRAIL alone, the fold increase in apoptosis would appear to be lower than actual. To correct for this, the ELISA results were normalized to cell counts.

Apoptotic death normalized to cell growth inhibition

The cell counts for BxPC3, MiaPaCa2 and Panc-1 cell lines reveal that the BITC treatments did impact cellular proliferation, confirming previous studies (39,63). When the Cell Death ELISA assay results were normalized to cell number, the data indicated that BITC significantly sensitizes all three human pancreatic cancer cell lines to TRAIL-induced apoptosis. BxPC3 cells underwent the greatest degree of apoptosis, as expected, as they are K-Ras 12 wild type. The K-Ras 12 mutant MiaPaCa2 cells had similar levels of apoptotic death. This suggests that the K-Ras 12 (gly→cys) mutation seen in MiaPaCa2 cells may not be dominant for chemotherapeutic resistance. Alternatively, there are likely to be several different mutations in these cell lines that in combination may determine overall resistance. Panc-1 cells were resistant to TRAIL-induced apoptosis but could be sensitized to TRAIL when treated in combination with BITC. The normalized Cell Death ELISA indicates that BITC is sensitizing mutant K-Ras 12 cancer cell lines to TRAIL-induced apoptosis.

Western Blot Analysis

Caspase 8 activation

Activation of caspase 8 was observed, as TRAIL is reported to signal through the death receptor pathway (72). For all three cell lines, overall there was a general decrease in caspase 8 proform levels going from vehicle, to BITC alone, to TRAIL alone and finally to BITC combined with TRAIL. BITC alone caused a substantial decrease in caspase 8 proform in BxPC3 cells but minimal change in MiaPaCa2 and Panc-1 cells, which could be because BxPC3 are K-Ras 12 WT. Interestingly, BxPC3 cells exhibited

minimal change in cleaved caspase 8 levels between TRAIL and BITC combined with TRAIL. This was unexpected as Cell Death ELISA results indicate these cells to be TRAIL sensitive and high levels of caspase 8 cleavage would be expected with TRAIL, which signals through the extrinsic cell death pathway. However, it may be that the high levels of apoptosis seen in BxPC3 cells are a result of increased activation downstream of caspase 8, such as through the intrinsic cell death pathway. The increased cleavage in MiaPaCa2 and Panc-1 cells suggests that BITC is sensitizing to TRAIL-induced apoptosis as early as initiator caspase 8.

XIAP cleavage

As a target of caspase 8, XIAP was examined (73,74). XIAP is an important prosurvival protein and its inactivation by caspase cleavage could allow for increased levels of apoptosis. XIAP levels had an overall decrease in BxPC3, MiaPaCa2, and Panc-1 cell lines going from vehicle, to BITC alone, to TRAIL alone, to BITC combined with TRAIL concurrently there was a substantial increase in the amount of XIAP cleaved in MiaPaCa2 and Panc-1 cells compared to BxPC3 cells. This with the normalized Cell Death ELISA results suggests that BITC may be sensitizing to TRAIL-induced apoptosis in the resistant K-Ras 12 mutant Panc-1 cells by decreasing XIAP levels. In MiaPaCa2 and Panc-1 cell lines, there is less XIAP cleavage with TRAIL alone compared to BITC alone, which supports research that has indicated TRAIL-resistance is related to maintained high levels of XIAP. High levels of XIAP have been implicated in TRAIL-resistance in pancreatic cancer cells previously. In MiaPaCa2 and Panc-1 cells, XIAP levels remained fairly constant compared to vehicle despite treatment with TRAIL.

However, upon treatment with BITC, XIAP levels are reduced and the Cell Death ELISA confirms increased apoptosis. One mechanism by which BITC is overcoming TRAIL resistance could be by inactivated prosurvival XIAP.

Bid cleavage

Bid cleavage was studied as it is also a direct target of caspase 8 and its truncated form can initiate the intrinsic cell death pathway (54). In BxPC3, MiaPaCa2, and Panc-1 cell lines there is a decrease in intact Bid levels between vehicle, BITC alone, TRAIL alone, and BITC combined with TRAIL. In both BxPC3 and MiaPaCa2 cells, there was a dramatic drop in Bid levels upon treatment with TRAIL alone compared to the gradual decrease of Bid levels observed in Panc-1 cells. This Bid truncation indicates that TRAIL is inducing both the extrinsic (death receptor) pathway but also suggests activation of the intrinsic (mitochondrial pathway). Initiation of apoptosis by dual apoptotic proteins could increase levels of apoptosis. The gradual loss of Bid in Panc-1 cells also illustrate that of these three cell lines, Panc-1 cells are the most resistant to TRAIL-induced apoptosis. The resistance seen in Panc-1 cells compared to the other cell lines evaluated may suggest that particular K-Ras 12 mutations may be associated with different levels of chemotherapeutic resistance. The complete loss of Bid observed with combined treatment of BITC with TRAIL suggests that BITC is sensitizing to TRAIL-induced apoptosis in Panc-1 cells.

Caspase 9 activation

Truncated Bid can lead to the activation of the intrinsic cell death pathway and caspase 9 (35). Caspase 9 activation was analyzed to determine the involvement of the intrinsic cell death pathway. All three cell lines showed an increase in cleaved caspase 9 going from vehicle, to BITC alone, to TRAIL alone, to BITC combined with TRAIL. Cleavage of caspase 9 confirms activation of the intrinsic cell death pathway. The most dramatic change was seen in BxPC3 cells, which supports these cells being TRAIL-sensitive. Interestingly, MiaPaCa2 cells showed only moderate increase in caspase 9 cleavage despite high levels of apoptosis observed in the Cell Death ELISA. However, this may be due to a cell specific pathway preference, meaning these cells may have preference of signaling from caspase 8 directly to caspase 3. This idea supported because despite low levels of caspase 9 cleavage, there are still high levels of caspase 3 cleavage and high levels of apoptosis as indicated by the cell death ELISA. Further analysis would be needed to confirm this pathway preference. Increased caspase 9 cleavage could be due to increased caspase 8 cleavage but BITC may be increasing cleavage by another mechanism. Previous research has shown BITC to increase the formation of ROS, which are capable of destabilizing the mitochondrial membrane (75). This may make the mitochondria more susceptible to other apoptotic stimuli and allow for increased TRAIL-induced apoptosis through the intrinsic cell death pathway.

Caspase 3 activation

Both the extrinsic and intrinsic cell death pathway lead to the activation of the main effector caspase, caspase 3 (35,55-60). In BxPC3, MiaPaCa2, and Panc-1 cells,

there was an overall decrease in caspase 3 inactive, proform levels from vehicle, to BITC alone, to TRAIL alone, and to BITC combined with TRAIL. For all cell lines, there was a gradual increase in cleaved caspase 3 levels. BxPC3 cells exhibited the greatest amount of caspase 3 cleavage, which supports these cells as being TRAIL-sensitive. MiaPaCa2 cells showed only slightly less cleavage of caspase 3 cleavage compared to BxPC3 cells. This is surprising as these cells are mutant K-Ras 12 and would therefore be expected to be TRAIL resistant. However, it may be that the type of amino acid change at K-Ras 12 is determinate of the level of chemotherapeutic resistance. Panc-1 cells as expected were resistant to TRAIL-induced cleavage of caspase 3, which suggests that the K-Ras 12 gly→asp mutation may greatly increase chemotherapeutic resistance compared to the K-Ras 12 gly→ cys mutation in MiaPaCa2 cells. However further study would be needed as there are many other different mutations in these cell lines that could interfere with response to chemotherapeutics. The levels of caspase 3 correspond to the levels of apoptosis observed in the normalized Cell Death ELISA.

PARP cleavage

Western blot analysis of cleaved PARP, a direct target of caspase 3, indicates the degree of caspase 3 activation. In BxPC3, MiaPaCa2, and Panc-1 cells, an increase in PARP cleavage was observed going from vehicle, to BITC alone, to TRAIL alone, to BITC combined with TRAIL. BxPC3 showed only moderate levels of PARP cleavage compared to the other cell lines, which is surprising as these cells are TRAIL-sensitive as indicated by high levels of caspase 3 activation and high levels of apoptosis as indicated by the Cell Death ELISA assay but these cells may have lower levels of PARP overall.

Interestingly, BITC alone substantially increased PARP cleavage in MiaPaCa2 cells, which conflicts with observed levels of caspase 3 cleavage. However, it may be that MiaPaCa2 cells have higher levels of PARP, which is unknown as only cleaved PARP was observed. Alternatively, the high levels of PARP cleavage may be attributed to cleavage by caspase 7, which can act in a similar manner as caspase 3. Panc-1 cells showed levels of cleaved PARP consistent to levels of caspase 3 activation. Cleavage of PARP, a known DNA repair enzyme, can leave cells less capable of repairing DNA damage and allow for apoptotic cell death.

Overall, NIH image analysis of Western blots for caspase 8, XIAP, Bid, caspases 9 and 3 and PARP confirm protein changes observed and support BITC combined with TRAIL increases cleavage of these proteins compared to TRAIL alone. These data taken together support the fact that BITC is sensitizing chemotherapeutically resistant pancreatic cancer cells to TRAIL-induced apoptosis.

Summary of findings

In conclusion, our results indicate that BITC sensitizes BxPC3, MiaPaCa2, and Panc-1 cells to TRAIL-induced apoptosis via dual apoptotic pathways and by inducing several key apoptotic proteins, namely caspases 8, 9 and 3 as well as there respective substrates XIAP, Bid and PARP. Results also show Panc-1 cells to be more resistant to TRAIL than BxPC3 and MiaPaCa2 cells, suggesting that certain K-Ras 12 mutations lead to increased TRAIL resistance, however further studies will be needed to confirm this observation. Our data suggests that BITC may be of value for the treatment of

chemotherapeutically-resistant mutant K-Ras 12 pancreatic adenocarcinomas and possibly other mutant K-Ras 12 tumors as well.

Future research

Panc-1 cells with a gly→asp K-Ras 12 mutation show a higher resistance to TRAIL induced apoptosis even with combined BITC and TRAIL treatment. The possibility exists that the gly→asp mutation leads to increased resistance to apoptosis.

This could be tested in several ways. The simplest of which would be to study TRAIL induced apoptosis in other pancreatic cancer cell lines with a K-Ras 12 gly→asp mutation. ASPC-1 cells are another pancreatic cancer with a K-Ras 12 gly→asp and would be ideal for this study. Another possible method, would be to point mutate a K-Ras 12 gly→asp mutation into a K-Ras 12 WT pancreatic adenocarcinoma cell line such as BxPC3. By using this method, all other mutations within the line would be unaffected and therefore any observations made would truly reflect the effects of the different K-Ras mutations. When using different cell lines, there are several other mutations that are different between each line that could potentially change levels of TRAIL sensitivity. Determining the effect of varying K-Ras 12 mutations or chemotherapeutic resistance could shed light on improved treatment methods for a largely untreatable disease.

K-Ras 12 mutations have been indicated in numerous other high impact cancers such as lung cancer, which is the leading cause of cancer related death in the United States, and colon cancer (3,4,17,76). In pancreatic cancer, there is a strong correlation to chemotherapeutic resistance with the presence of mutated K-Ras. These same mutations

may play a similar role in treatment resistance of other cancers. Again, it would be important to determine if specific K-Ras 12 mutations are related to varying resistance.

Although K-Ras is largely associated with the aggressiveness and chemotherapeutic resistance in pancreatic genes, other alterations within P53, Smad4, and PI3K signaling have been implicated in tumor invasiveness and resistance (12,77,78). PI3K is an upstream regulator of NF- KB, which has been implicated in chemotherapeutic resistance in pancreatic cancer (49,50). NF-KB can upregulate the expression of XIAP, which is a prosurvival factor (79). Previous research has indicated that TRAIL resistance in pancreatic cancer is partially attributed to both XIAP and NF-KB (49,50). RNA interference of XIAP and NF-KB has been shown to increase TRAIL sensitivity in resistant pancreatic cancer cell lines (49,50). Although it is known that Ras signals through PI3K and subsequently NF-KB, it is not yet known that this is the pathway by which K-Ras mediates chemotherapeutic resistance. PI3K is downstream of Ras and it may be possible that K-Ras mutations and/or mutations within PI3K are involved in resistance mediated by NF-KB. Ras signals through PI3K to regulate NF-KB, which in turn upregulates expression of XIAP, which is a prosurvival factor and is capable of inhibiting caspases (80,81). K-Ras 12 mutations may contribute to resistance of XIAP cleavage, which has been observed in previous studies as well as this one (49). This may occur by increased signaling through PI3K, which is an upstream modulator of NF-KB and subsequently XIAP (80,81).

As a continuation of this project, PI3K and NF-KB should be studied to determine if they are in fact involved in the resistance seen in K-Ras 12 mutated cells. BxPC3 (K-Ras 12 WT), MiaPaCa2 (K-Ras 12 gly→cys), and Panc-1 (K-Ras 12 gly→asp) cell lines

would be ideal to use as they represent common K-Ras 12 mutations and range from being TRAIL-sensitive to TRAIL-resistant. In this study, it appeared that BITC led to a greater decrease of XIAP levels in both MiaPaCa2 and Panc-1 cells than TRAIL did. If this is confirmed, this may reveal how BITC is sensitizing these cells and in the following experiments BITC should be tested to observe any inhibition of PI3K and NF-KB, which would decrease XIAP levels. NF-KB activity levels should be analyzed using a Trans-AM ELISA for p50 and p65 NF-KB in the presence of vehicle, BITC alone, TRAIL alone, BITC combined with TRAIL, a pan-NF-KB inhibitor alone, and finally the NF-KB inhibitor with TRAIL. If BITC treatment reduces NF-KB activity, this would suggest that sensitization by BITC is a result of leading to the decrease of prosurvival XIAP. If NF-KB is responsible for resistance, the NF-KB inhibitor should restore sensitivity in the resistant cell lines. Higher NF-KB activity levels in the resistant cell lines would support its responsibility for the observed TRAIL resistance. It also suggests that with further study, this may be the mechanism by which K-Ras 12 is modulating chemotherapeutic resistance. To confirm this, the pathway needs to be traced back to K-Ras. PI3K is the upstream modulator of NF-KB. To determine PI3K's involvement in increased NF-KB activity and not some other modulation of NF-KB, a time course study of both PI3K and NF-KB by Western blot analysis would be required. If PI3K is involved, Western Blot analysis should show PI3K activation before increased activity of NF-KB. If PI3K activation is observed before NF-KB activation, then cells treated with a PI3K inhibitor such as LY294002 should show decreased levels of NF-KB activity and possibly decreased levels of XIAP. Resistant cell lines should become TRAIL-sensitive upon treatment with a PI3K inhibitor, if this is how resistance is acquired. This would be

determined by quantifying apoptosis by an assay such as the cell death ELISA. This increased signaling through PI3K could be a result of K-Ras, however there are many other factors that stimulate this pathway. If increased signaling was observed in the K-Ras 12 mutant cell lines such as MiaPaCa2 or Panc-1 cells as compared to K-Ras 12 WT BxPC3 cells, this would further implicate K-Ras 12 as the initiating factor. To confirm that the involvement of K-Ras 12 in increased PI3K signaling, further research would be needed. If inhibition of K-Ras led to a decrease in PI3K signaling and subsequently restored TRAIL-sensitivity, this taken with the results of the above NF-KB and XIAP experiments could confirm that one way K-Ras 12 is modulating chemotherapeutic resistance is through PI3K. K-Ras activity could be inhibited by knock down of K-Ras 12 by viral tranfection of shRNA against the specific amino acid mutations observed in MiaPaCa2 and Panc-1. Previous studies have successfully used adenoviral delivered K-Ras sirna in lung cancer cells (82). K-Ras 12 has been successfully inhibited by transfection of siRNA in previous studies (83). However, shRNA would allow for a more stable knockdown of a gene and with an adenoviral delivery system can remain episomal to prevent permanent interference with the genome (84,85,86). This should at minimum be carried out in the sensitive K-Ras 12 WT BxPC3 cells and the resistant K-Ras 12 gly→asp Panc-1 cells. Cells should be treated with vehicle, TRAIL alone, a scrambled shRNA, a shRNA against K-Ras 12 G12D and TRAIL combined with shRNA against K-Ras 12 G12D. Levels of K-Ras 12 observed by Western blot analysis would confirm if K-Ras was successfully knocked down. Decreased levels of activated PI3K in the knockdowns would confirm that K-Ras 12 is modulating PI3K activity. Apoptosis should be quantitated to determine if knockdown of K-Ras restored TRAIL-sensitivity in

the resistant Panc-1 cell line. Taken together, information from these experiments could shed light as to how K-Ras 12 mutations confer TRAIL resistance and allow for the formulation of more effective therapeutics.

V. APPENDIX

Abbreviations

Asp-Aspartate

BITC-Benzyl Isothiocyanate

Cys-Cystine

DR–Death receptor

DCR-Decoy Receptor

ELISA-Enzyme-Linked ImmunoSorbent Assay

H-Ras-Harvey Ras

GDP-Guanine Diphosphate

Gly-Glycine

GTP-Guanine Triphosphate

K-Ras- Kirsten Ras

N-Ras-Neuroblastoma Ras

NF-Kb-Nuclear Factor kappa B

OPG-osteoprotogerin

PARP- Poly (ADP-ribose) polymerase (PARP)

PBS-Phosphate Buffered Saline

PI3K-Phosphoinositide 3-kinases

SDS-PAGE-Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis

TBS-Tris Buffered Saline

TNF- Tumor Necrosis Factor

TRAIL- TNF related Apoptosis Inducing Factor

TGF-beta- Transforming Growth Factor beta

Val-Valine

XIAP- X linked Inhibitor of Apoptosis

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